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Review

Gene therapy and gene editing strategies for hemoglobinopathies

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ABSTRACT

Gene therapy for hemoglobinopathies is currently based on transplantation of autologous hematopoietic stem cells genetically modified with an integrating lentiviral vector expressing a globin gene under the control of globin transcriptional regulatory elements. Studies and safety works demonstrated the potential therapeutic efficacy and safety of this approach, providing the rationale for clinical translation. The outcomes of early clinical trials, although showing promising results, have highlighted the current limitations to a more general application. These include the nature, source and age of repopulating hematopoietic stem cells, the suboptimal transduction efficiency and gene expression levels, the toxicity and efficacy of bone marrow conditioning, the stress status of bone marrow microenvironment in chronic diseases such as β-thalassemia and sickle cell disease. Recently, gene editing strategies based on the use of nucleases offered a novel approach to increase globin expression in a quasi-physiological way, independently from the addition of transgenes and viral sequences to the human genome. This review will discuss the current status of gene therapy for β-thalassemia and sickle cell disease with a perspective towards the improvements necessary in the context of clinical translation.

1. Introduction

Hemoglobinopathies are the most common genetic diseases that afflict humans worldwide [1,2]. The most common forms of hemoglobinopathies are thalassemia and sickle cell disease (SCD). Thalassemia is a quantitative disorder characterized by a decrease in the α-globin chain (α-thalassemia) or in the β-globin chain (β-thalassemia) production, whereas SCD is a qualitative disorder in which there is a structural variant in the hemoglobin (Hb) molecule. The majority of basic and clinical research focuses on β-thalassemia and SCD. SCD is the most diffuse inherited hemoglobinopathy worldwide, occurring in 330,000 births annually worldwide [2]; roughly 75% of these births are in Sub-Saharan Africa [3]. In the United States, SCD affects > 100,000 Americans [4].

SCD is caused by an A - T transversion in the adult β-globin gene, which leads to a substitution of a valine for a glutamic acid at position 6 in the β-globin chain [5]. The dimerization of these mutant β-globin chains with α-globin results in the formation of HbS (α2β2S). In patients homozygous for HbS, the most severe form of SCD, the mutant β-globin chains polymerize in deoxygenated red blood cells (RBCs), causing them to deform, from a biconcave morphology into the hook/sickle shape. Repetitive cycles of sickling, as HbS molecules switch from oxygenated to deoxygenated states, causes RBC fragility and promotes vaso-occlusion, painful crises, chronic anemia, acute chest syndrome, organ failure, stroke, and eventually death [6,7]. Current treatments include chronic blood transfusions and hydroxyurea, an inducer of fetal hemoglobin (HbF) synthesis, as the only FDA (Food and Drug Administration) approved drug for this disease. Hydroxyurea kills erythroid cycling precursors by inhibiting ribonucleotide reductase and affects the production of red cells containing a high level of sickle hemoglobin. In addition, hydroxyurea treatment stimulates directly expression of γ-globin genes by producing nitric oxide and induces the production of red cells containing a high fetal hemoglobin level [8,9]. Despite improvements in supportive care, disease morbidities remain severe and life expectancy significantly shortened [8,10].

A related hemoglobinopathy with high global incidence is β-thalassemia, occurring in 68,000 births annually worldwide [2]; 93% of newborns with β-thalassemia are in Asian, Indian, and Eastern Mediterranean regions [2]. As a result of changing demographics, β-thalassemia is becoming increasingly more common in North America and Europe [11]. The molecular basis of β-thalassemia is caused by point mutations or deletions in HBB gene resulting in a reduction (β + genotype) or complete elimination (β0 genotype) of β-chains from HbA, chains imbalance with excess of α-chains, and consequently ineffective erythropoiesis. Although protecting against malaria in heterozygous carriers, homozygosity of β-thalassemia mutations results in a disease phenotype with varying severity, depending on the type of mutations and other modifying factors, from mild forms of anemia to severe

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anemia referred to as β-thalassemia major [6,12]. In addition to chronic hemolytic anemia, patients affected by β-thalassemia major show disease hallmarks as compensatory hematopoietic expansion in bone marrow (BM) cavities, often resulting in bone deformities, and in ectopic sites, hypercoagulability and increased intestinal iron adsorption (reviewed in [13]). The conventional management for patients affected by the severe forms of the disease relies on chronic and regular blood transfusions in association to iron-chelation therapy.

Both for SCD and β-thalassemia, the only curative therapy is represented by allogeneic hematopoietic stem cell transplantation (HSCT), with a disease-free survival exceeding 80% up to 93%, depending on patient age and clinical status [14–16]. Nevertheless, the availability of a well-matched donor is critical for a favorable outcome. There is therefore an unmet medical need for alternative curative treatments with the potential to provide long-term benefit, and gene therapy has been proposed as theoretically feasible in a large number of patients, since it relies on an autologous transplant with no need for a matched donor. The focus of this review is to describe the principles and the limitations of gene therapy for hemoglobinopathies, the critical steps necessary for a successful clinical translation and preliminary results of the ongoing trials.

2. Gene therapy by gene addition

2.1. Efficacy studies

Gene therapy for genetic blood diseases is currently based on transplantation of autologous hematopoietic stem cells (HSCs) genetically modified with integrating viral vectors expressing the transgene of interest (Fig. 1A and B). In the case of SCD and β-thalassemia, gene therapy presents additional challenges in respect to other diseases, in part due to the complexity of β-globin gene regulation (reviewed in [17] of this issue), which imposes constraints to the development of efficient vectors. Studies of the gene therapy approach for treating hemoglobinopathies have been made possible by the availability of transgenic and knock-out murine strains models for SCD [18] [19] and β-thalassemia [20,21]. Earlier gene therapy studies utilized recombinant γ-retroviral vectors (RV), derived from the Moloney Leukemia Virus (MLV), as carriers to introduce a functional copy of the β-globin gene, but a decade long investigation with these vectors resulted in: a) variable and low level expression of the β-globin transgene [22,23], b) unstable vectors with multiple rearrangements [24], and c) vectors with low titers. The incorporation of the core elements of the hypersensitive sites (HS) 2, 3, and 4 of the human β-globin locus control region (LCR) was instrumental to increase β-globin expression. However, it failed to prevent vector rearrangements and position-effect variegation, and to achieve therapeutic levels of β-globin expression [25,26]. In the mid-1990s, lentiviral vectors (LV) were derived by HIV and offered an alternative to oncoviral vectors as possible shuttles of the β-globin gene [27].

2.1.1. Gene therapy studies on β-thalassemia

Several laboratories have shown that self-inactivating LVs, carrying the human β or γ-globin gene (named TNS9, sGbG, HPV569, BB305, GLOBE and others, see Table 1) and its fundamental regulatory elements, are able to correct the disease in animal models for β-thalassemia intermedia and major [28–32]. A continuous effort was pursued by several investigators to improve LV in terms of higher transgene expression, acting mostly on regulatory transcriptional components and adding insulators to prevent negative chromatin position effects. Further optimization of the LCR components included larger HS2 and HS4 elements of LCR, incorporation of insulator elements, the inclusion of HS1 or GATA-1 elements [33–36]. In most cases increased transgene expression was achieved at the cost of detrimental effect on vector titer, thus lowering transduction efficiency and vector copy number/cell (VCN). Increasing the transgene size inserted between the LTRs can influence the packaging efficiency thus inducing a reduction in viral titers [37]. Therefore, the final LVs entering the approval for ongoing clinical trials were the result of an acceptable compromise among good manufacturing yield, titer and β-globin expression level.

Long-term correction of thalassemia with LV-transduced HSCs in a murine model of thalassemia intermedia was first reported in the seminal work by May and colleagues [28]. Major advantages of globin LVs with respect to their RV predecessors were higher transduction efficiency in long-term repopulating HSCs and stable and persistent gene expression, even after serial HSC transplantation in mice. Results from *in vivo* gene correction allowed establishing the minimal dose of transduced cells and VCN to be therapeutic in the murine models. Later, it was shown that genetically corrected erythroblasts undergo *in vivo* selection in mice, indicating that the corrected cells have a survival advantage over the thalassemic ones [32]. This result supports the evidence that low levels of donor engraftment can result in significant functional improvement for patients with persistent mixed chimerism after allogeneic donor BM transplantation [38]. From a clinical point of view, this advantage could imply a lower myeloablative regimen for a patient, who could benefit from the engraftment and the persistence of even a proportion of corrected HSCs. On this assumption, it is based the rationale of gene therapy, since the co-existence of gene corrected cells with those still expressing the genetic defect will be an expected scenario also in an autologous environment.

In addition to the results obtained in thalassemic mutant mice, LV-mediated correction of human cells from thalassemic patients was shown, adopting models of *in vitro* human erythropoiesis and in the xenograft model of immunodeficient mice transplanted with human cells [39,40]. Roselli et al. [41] proved that the *ex vivo* manipulation of HSCs with a LV does not negatively affect the functional capacity of thalassemic $CD34^+$ cells and their global gene expression profile. Moreover, they showed that relatively low VCN is able to correct the deficiency of adult globin expression restoring the erythropoiesis *in vitro*. Finally, they demonstrated that LV has preferences for genomic integration sites similar to those observed for other LVs, with no bias towards integration in the proximity of proto-oncogenes. This work represents the first translational study that describes the pre-clinical evaluation of the treatment of β-thalassemia by using LV in human cells. These data supported the clinical trial application to regulatory agencies for further clinical translation (see Section 2).

2.1.2. Gene therapy studies on SCD

Different recombinant hemoglobins were designed to inhibit HbS-polymerization upon de-oxygenation. It was reported that β-globin chains containing alanine at position 22 and/or threonine at position 87 significantly block deoxy-HbS polymerization by disrupting the formation of axial and lateral contact between Hb tetramers [42]. Transplantation of BM cells transduced with LV carrying anti-sickling Hb variants and β-LCR resulted in correction of the pathology in SCD mouse models. In the study by Pawliuk et al. [43] the βA-T87Q-globin variant, having a threonine to glutamine mutation at codon 87, was cloned into a LV structurally optimized for erythroid-specific expression. Transplantation of LV-transduced BM from SCD mice into lethally irradiated recipients resulted in up to 52% of total Hb consisting of the βA-T87Q variant with correction of RBC and reticulocyte counts, amelioration of splenomegaly and urine concentration defect. Levasseur et al. [44] demonstrated comparable results using a LV containing a different anti-sickling human Hb variant (βAS3-globin), carrying additional amino acid substitutions of aspartic acid, alanine and threonine at positions 16, 22 and 87, respectively. The substitution at position 16 confers the recombinant β-globin subunit (βAS3) with a competitive advantage over βS for interaction with the α-globin polypeptide. Romero et al. showed that the insulated βAS3-FB LV transduce efficiently human BM-derived $CD34^+$ HSCs from SCD patients, leading to expression of an effective anti-sickling β-globin gene [45]. Pestina et al. [46] showed correction of SCD phenotype by increasing the expression of HbF using γ-Globin LVs (V5m3). However, no correlation between VCN in the BM cells and the amount of HbF was observed. Mice with lower VCN showed HbF levels equivalent to those with

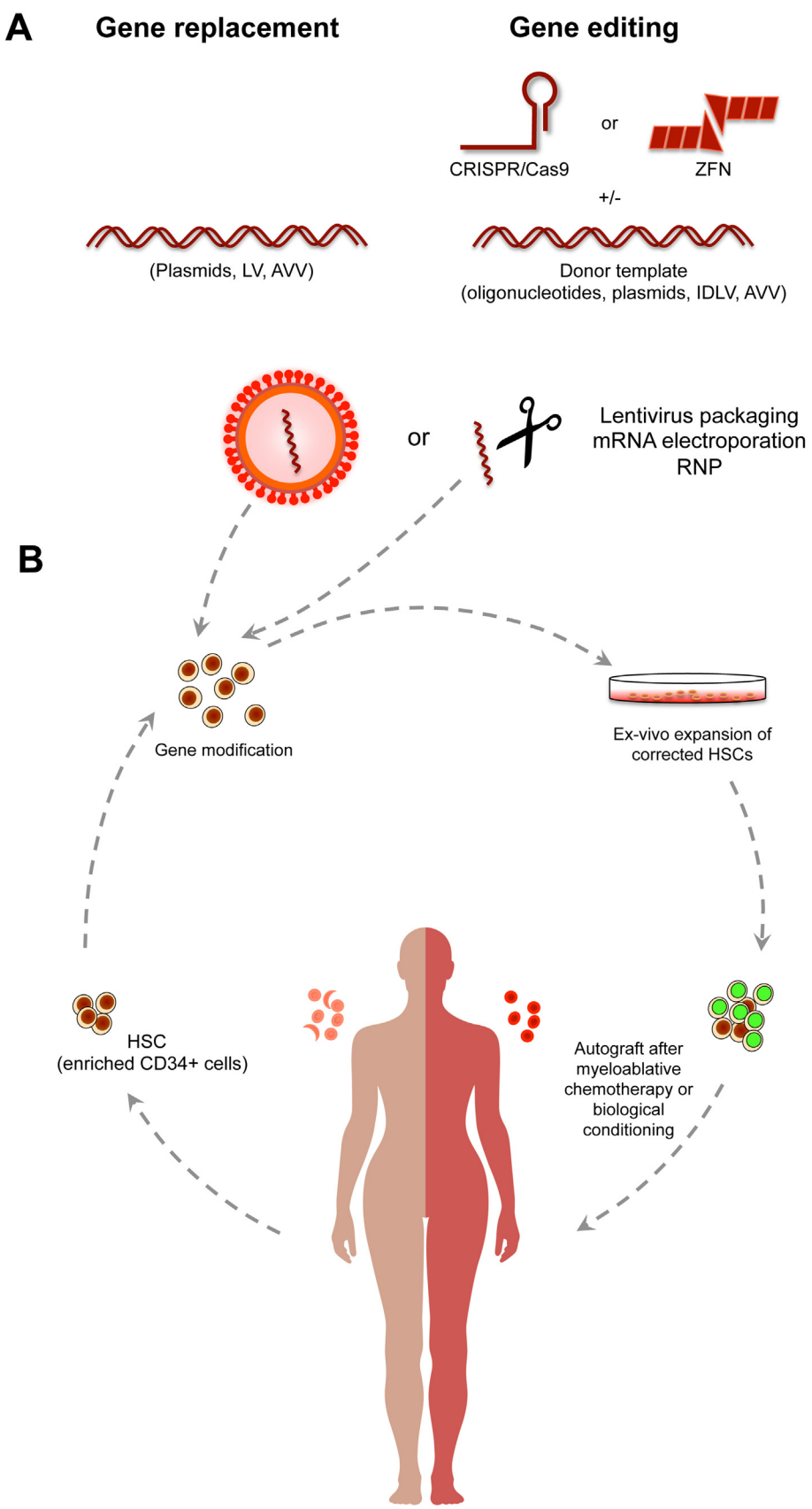

Fig. 1. Strategies of gene therapy for hemoglobinopathies.
A. Molecular tools used in gene therapy by gene replacement and by gene editing.
B. Scheme representing the stepwise procedure of gene therapy: autologous transplantation of cells genetically modified by gene replacement or gene editing in patients affected by β-thalassemia or sickle cell disease.
Nuclease are represented by a scissor.
LV: lentiviral vector; AAV: adeno-associated vector; ZFN: zinc finger nuclease; IDLV: integrase defective lentiviral vector; RNP: ribonucleoprotein.

Table 1
Clinical trials for β-thalassemia.

ClinicalTrials.gov Identifier/other study number/phase	Transgene LV vector	Country	Sponsor	Start date	Enrollment n./age patients	Conditioning regimen	Cell source	Cell dose	n/Age treated patients	VCN DP	References
NCT01639690 Phase I	β-globin TNS9.3.55	USA	MSKCC	July 2012	10/≥ 18 yrs	Busulfan: 8 mg/kg	G-CSF PB	11.8–8.4 × 10^6 $CD34^+$ cells/kg	4/ 18–23 yrs	0.39–0.21	[71]
LG001 study Phase I/II	$\beta^{A\text{-}T87Q}$- globin HPV569	France	Bluebird bio (formerly Genetix Pharmaceuticals)	Sept, 2006	5 β-Thal 5 SCD 5–35 yrs	Busulfan: 12.8 mg/kg, pk-adjusted	BM G + Plx PB	3.9 × 10^6 $CD34^+$/kg 4.3 × 10^6 CD34 cells/kg	2/18 yrs 1/22 yrs	0.6 0.3	[53]
NCT02151526 HGB-205 study Phase I/II	$\beta^{A\text{-}T87Q}$- globin BB305	France	Bluebird bio	August 2013	7/5–35 yrs	Busulfan: 12.8 mg/kg, pk-adjusted	G + Plx PB	8.8–13.6 × 10^6 $CD34^+$/kg	4/16-18 yrs	0.8–2.1	[69,70] http://www.businesswire.com/news/home/20170623005225/en/bluebird-bio-Presents-New-Data-HGB-205-Study
NCT01745120 HGB-204 study Phase I/II (Northstar)	$\beta^{A\text{-}T87Q}$- globin BB305	USA, Thailand, Australia	Bluebird bio	August 2013	18/12–35 yrs	Busulfan: 12.8 mg/kg, pk-adjusted	G + Plx PB	5.2–18.1 × 10^6 $CD34^+$/kg	18/ 12–35 yrs	0–3.1.5	[67,68]
NCT02906202 HGB-207 study Phase III (Northstar-2)	$\beta^{A\text{-}T87Q}$- globin BB305	USA, Europe Thailand, Australia	Bluebird bio	July 2016	15/12–50 yrs	Busulfan: 12.8 mg/kg	G + Plx PB	7–8.1 × 10^6 $CD34^+$/kg	3/ 20–22 yrs	2.4–3.2	22nd EHA 2017 http://www.businesswire.com/news/home/20170623005066/en/bluebird-bio-Announces-Early-Data-Phase-3
NCT02453477 (TIGET-BTHAL) Phase I/II	β-globin GLOBE	Italy	Telethon Foundation	May 2015	3/≥ 18 yrs 3/8–17 yrs 4/3–7 yrs	Treosulfan 42 g/m2 + thiotepa 8 mg/kg	G + Plx PB	16–19.5 10^6 $CD34^+$/kg	3/31-35 yrs 3/13 yrs 1/6 yrs	0.7–1.5	[73]

higher VCN. These results are consistent with other works that highlighted the impact of position effect on globin LV-derived expression [30,43,47]. In a transplantation model for SCD, 20% HSC correction has been demonstrated to be sufficient for a significant amelioration of sickling, organ damage, and survival [48]. However, it is difficult to predict whether the clinical benefit for SCD is achievable with this percentage of gene-modified HSCs also in humans.

2.2. Safety studies

In addition to the proof of efficacy, gene therapy preclinical studies are aimed to demonstrate safety in order to support clinical protocol applications. The studies must be compliant with the regulatory requirements listed in the guidelines across countries and continents, that might vary but all aim to ensure safety of the use of gene therapy medicinal products (GTMP) (reviewed in [49]).

The globin vectors combine the reduced genotoxic properties of LVs to the strict lineage specificity of their transcriptional regulatory elements (β-globin promoter, enhancer and LCR), which have only minimal, if any, capacity to activate neighboring promoters in stem and progenitor cells. Different combination of LCR elements have been tested in LV vectors, in an attempt to improve vector titer and infectivity, a persistent problem due to the size but also the complex nature of the β-globin locus regulatory sequences. To further improve safety of globin LVs, several investigators inserted chromatin "insulators" in the LTRs, with the intent of shielding the vector from the repressive effect of neighboring chromatin, or the promoter of neighboring genes from any influence of the LCR enhancers. Examples of such elements are the chicken β-globin HS4 [36,50] or a synthetic element (FB) that combines sequences from the chicken HS4 and the T-cell receptor BEAD-1 insulator [45,51]. Desprat et al. [52] analyzed the effect of integration of globin therapeutic cassettes in the presence or absence of the chicken HS4 and three other putative insulators inserted near three cellular proto-oncogenes, such as Stil, Tal1 and MAP17, by using used Recombinase-Mediated Cassette Exchange (RMCE). Unexpectedly, results from this study showed variability of insulator-mediated suppressive effect on activation of nearby genes. Unfortunately, the presence of insulators in the LTR led in most cases to decrease of vector titer, low transduction efficiency and genetic instability with partial loss of LTR sequences. In particular, the chicken HS4 insulator, inserted in tandem configuration in the LV used in the first clinical trial of gene therapy for β-thalassemia (HPV569), revealed a cryptic splicing activity that triggered abnormal splicing at the insertion site in the proto-oncogene HMG2A and a consequent benign clonal dominance in one of the treated patients [53]. Moiani et al. [54], confirmed that β-globin carrying LV may induce aberrant RNA splicing leading to the formation of chimeric transcripts harboring HIV sequences fused to cellular exon sequences in hematopoietic cells. However, the abundance of these aberrant transcripts is limited by nonsense-mediated mRNA degradation.

The β-globin HPV569 LV was further improved for safety by removing the insulator domains, found to be unstable, and generating the derived BB305 LV. In a murine BM transplant study, BB305 LV displayed preferred integration into gene coding regions with 51.9% of integrations found in common insertion site regions, which have not previously been associated with adverse events in patients. Preferred insertional integration was not observed in high-risk genes LMO2 or MDS1-EVI1, associated with transformation, in primary and secondary transplanted mouse recipients [55]. Moreover, no evidence of LV-associated tumors was found. Phase 1/2 clinical trials are currently in progress for gene therapy of β-thalassemia and SCD using the BB305 LV (see below).

To evaluate the safety of GLOBE LV, *in vivo* studies of tumorigenicity, toxicity and biodistribution were conducted in the GLP (Good Laboratories Practice) setting. Murine HSCs were transduced with GLOBE LV and transplanted in thalassemic *th3/+* mice, conditioned by busulfan, using cell dose and VCN superior to that expected in humans. After 1-year follow-up, this study showed absence of vector induced

toxicity and tumorigenicity, as detectable over a background of spontaneous tumor incidence. The biodistribution study, conducted by transplanting human GLOBE LV- or mock-transduced CD34$^+$ cells in immunodeficient NSG mice, demonstrated that transduced cells successfully engraft and generate myeloid and lymphoid progenies comparably to control cells and in absence of adverse events [56] (Lidonnici et al. in preparation).

In the context of SCD, recent *in vivo* toxicology studies with βAS3-FB LV did not revealed any toxicity from the lentiviral vector [57]. Other unpublished works have been conducted to support clinical trial application with LVs in SCD

3. Clinical trials of gene therapy for β-thalassemia and SCD

Over the past two decades, gene therapy with autologous HSCs using RV or LV has been successfully applied to primary immunodeficiencies, such as adenosine deaminase-deficient severe combined immunodeficiency (ADA-SCID), X-linked SCID (SCID-X1), Wiskott-Aldrich syndrome (WAS) and X-linked chronic granulomatous disease (X-CGD) (reviewed in [58,59]) and storage disorder (ALD, MLD). While the use of RVs has been associated with a significant risk of oncogenesis in SCID-X1, CGD and WAS clinical trials [60–64], LVs have been so far demonstrated to be nontoxic in several *in vitro* and animal models and they are currently used in early- or advanced-phase clinical trials of gene therapy for WAS, ADA-SCID, X-CGD, ALD, MLD, β-thalassemia, SCD and HIV-associated lymphoma (reviewed in [65]).

The list of current gene therapy trials for β-thalassemia and SCD is shown in Tables 1 and 2.

In June 2007, in the first trial for β-thalassemia in France, three patients were treated by gene therapy with the HPV569 LV expressing the β^{T87Q} globin variant. One year after gene therapy, one patient affected by transfusion-dependent HbE/β-thal, he became transfusion independent with hemoglobin levels of 8.5 to 9 g/dL, contributed in almost equal proportions by the vector, elevated HbF synthesis and the HbE allele. Integration site analysis revealed expansion of a single clone in which the provirus was inserted in the *HMGA2* (high-mobility-group AT-hook 2) proto-oncogene. The gene was activated by a vector-induced abnormal splicing and premature transcript termination, caused by a cryptic splice site in the vector cHS4 insulator. The benign clonal dominance persisted for almost 9 years, after which it started to decline and currently contributes for < 10% of the circulating nucleated cells. The patient maintains stable though low levels of therapeutic hemoglobin, and requires occasional transfusion [66].

Further improvements of the original LV led to subsequent clinical trials, such as the multicenter Northstar HGB-204 study [67,68] and the single-center HGB-205 study [69,70], all based on the BB305 vector and ongoing in USA, Australia, Thailand, and France (Table 1). As of June 2017 (EHA 2017), of all patients with genotypes different from β^0/β^0 and a follow-up of ≥ 12 months (n = 14), twelve patients have discontinued transfusions. Two patients with non-β0/β0 genotypes who still require intermittent transfusions had annual transfusion volumes reduced by 30% and 94%. Of all patients with β^0/β^0 genotypes (n = 8), six still require transfusions, although with requirements decreased to 60% (median value) with respect to baseline. Two patients with β0/β0 genotypes have not received a transfusion in more than a year. In both studies, gene therapy was well tolerated, with no gene transfer-related severe adverse event. Very recently, 2 different phase 3 trials (HGB-207 and HGB-212) have been opened for non-β0β0 and β0β0 transfusion dependent β-thalassemia patients, respectively. Initial data from the HGB-207 (Northstar-2) trial will be reported at ASH annual meeting 2017. At time of this writing this review, abstract was released on ASH website. Three patients were treated and had successful engraftment. The first patient treated has been transfusion-free for 5 months.

In 2012, a trial opened at Memorial Sloan Kettering Cancer Center (MSKCC) in New York and four patients have been treated with gene therapy using the TNS9.3.55 LV. The need for full myeloablation

Table 2
Clinical trials for sickle cell disease (SCD).

ClinicalTrials.gov Identifier/other study number/phase	Transgene LV vector	Country	Sponsor	Start date	Enrollment n/ age patients	Conditioning regimen	Cell source	Cell dose	n/Age treated patients	VCN DP	References
NCT02151526 HGB-205 Phase I/II	$\beta^{A\text{-}T87Q}$-globin BB305	France	Bluebird Bio	July 2013	7/5–35 yrs	Busulfan: 12.8 mg/kg, pk-adjusted	BM	3–5.6 × 10^6 CD34 + /kg	3/13–21 yrs	0.8–1.2	[69–70] http://www.businesswire.com/news/home/20161203005050/en/
NCT02140554 HGB-206 Phase I + Amendment[a]	$\beta^{A\text{-}T87Q}$-globin BB305	USA	Bluebird Bio	August 2014	29/ ≥ 18 yrs	Group A Busulfan [a]Group B Busulfan: 12.8 mg/kg, pk-adjusted [a]Group C Busulfan: 12.8 mg/kg, pk-adjusted	BM [a]Plerixafor mPB	1.6–5.1 × 10^6 CD34 + /kg 2.2–3.2 × 10^6 CD34 + /kg [a]Planned increased cell dose	7/18-42 yrs 2 1	0.3–1.3 [a]1.4–5 –	[77,78] [78] http://www.businesswire.com/news/home/20171101005861/en/ http://investor.bluebirdbio.com/news-releases/news-release-details/bluebird-bio-announces-updated-clinical-results-ongoing-phase-0
NCT02247843 Phase I	βAS3 globin βAS3-FB	USA	Donald B Kohn, MD, UCLA	July 2014	6/ ≥ 18 yrs	Busulfan: 12.8 mg/kg, pk-adjusted	BM	4 × 10^6 CD34 + /kg	1/25 yrs	0.3	Personal communication
NCT02186418 Phase I/II	γ-globin mLARβΔ↑V5	USA	Cincinnati Children's Hpt	July 2014	10/18–35 yrs	Melphalan 140 mg/m^2	BM	–	–	–	N.A.

[a] Amendment of HGB-206 clinical protocol.

emerged from this study, where partially myeloablative conditioning led to insufficient gene marking with minimal clinical benefit [71].

More recently, in 2015, a phase I/II gene therapy trial with the GLOBE LV started in Italy (TIGET BTHAL) for adult and pediatric patients affected by transfusion dependent β-thalassemia. A reduced intensity and toxicity conditioning including treosulfan and thiotepa was adopted. Treosulfan has already been used in cohorts of thalassemic pediatric and adult patients in the context of allogeneic HSCT showing reduced toxicity when compared with busulfan [72]. Peripheral blood stem cells are harvested following mobilization with the lenograstim and plerixafor. After transduction of immune-selected autologous $CD34^+$ cells and successful release of the cryopreserved drug substance, gene modified $CD34^+$ cells are administered to the patient. The route of administration of gene modified HSCs is intraosseous in the posterior-superior iliac crests with the aim of enhancing engraftment and minimizing first-pass intravenous filter. After 2 years follow-up, patients will be followed up for a further six years in a long-term follow-up study (NCT03272051, GSK 207757). The clinical study foresees treatment of 10 patients: 3 adults (group 1) followed by 3 patients aged 8–17 years (group 2) and 4 patients aged 3–7 years (group 3), with a staggered enrolment strategy based on evaluation of safety and preliminary efficacy in adult patients by an independent data safety monitoring board before inclusion of pediatric subjects. As of September 2017, 7 patients (3 adults, 3 adolescents and 1 pediatric) have been treated. The procedure was well tolerated, the adverse events experienced by patients were consistent with the conditioning regimen, and no gene therapy product related adverse events have been reported to date. Multilineage engraftment of gene-marked cells was observed in all tested patients, with polyclonal vector integrations profiles in the first analyzed patients and no evidence of clonal skewing. The clinical outcome indicates so far significant reduction in transfusion requirement and improved quality of life in adult patients and greater clinical benefit in younger patients [73] (and unpublished results).

Clinical trials for SCD with BBB305 LV (HGB-205, HGB-206), currently running in France and in the USA are based on bone marrow harvest (BMH), since previous attempts to mobilize HSPCs by low-dose G-CSF treatment caused severe adverse events and one fatality and is no longer used [74]. Three SCD patients have been treated in HGB-205 study, with one showing early clinical benefit. He received full myeloablative chemotherapy and was transplanted with 5×10^6 $CD34^+$ cells/kg selected from 2 BMH and transduced at an average VCN of 1. No drug product related adverse events and no clonal abnormalities were observed on a 2-years follow-up. The patient achieved a level of therapeutic $\beta^{A\text{-}T87Q}$ globin around 50% and transfusion independence, with a clinical picture comparable to that of a HbS carrier [70,75,76].

Other 7 SCD patients have been treated in the HGB-206 multicenter USA trial and underwent cell procurement with a median of 2 BMH and a median VCN of 0.6 [77]. However, these patients failed to achieve a level of correction comparable to that observed in the patient of HGB-205 study, showing a rapid decrease in VCN in peripheral blood cells. Investigations of possible causes of lack of success are ongoing. These results pointed to the difficulty in obtaining adequate doses and robust engraftment of transduced HSCs in SCD patients. Stem cell procurement, transduction efficiency and patient conditioning appear the most critical factors that need improving in order to achieve clinical benefit. So far, two patients were treated with the improved drug product. Early results demonstrate both higher DP VCN and higher peripheral VCN after transplant [76,78].

In the evolution of HGB-205 and HGB-206 studies for SCD, it has been planned to change the $CD34^+$ cell source from BM to plerixafor mobilized peripheral blood, with the purpose of increasing cell harvest [76,79].

Two more trials have recently opened in the USA based on the use of LVs expressing the βAS3 or the γ-globin (Table 2).

4. Critical issues related to gene therapy for SCD and β-thalassemia

Clinical gene therapy outcome depends on different variables including the source of HSCs, efficiency of transduction (as percentage of transduced cells) and the VCN achieved during *in vitro* transduction and persisting *in vivo*, intensity of myeloablation, dose and engraftment of genetically modified HSCs infused, and eventually the BM microenvironment status. The relevance of these aspects is discussed in the following sections.

4.1. Source of hematopoietic stem/progenitor cells

HSCs reside in the bone marrow niche but can be enforced to egress into the blood, a process termed mobilization. Mobilized peripheral blood has been considered the standard approach for collecting hematopoietic stem cell in the majority of autologous and allogeneic transplantations. This procedure provides several-fold higher numbers of HSCs over bone marrow harvest. Gene therapy protocols for genetic disorders use autologous purified $CD34^+$ cells as starting product to be transduced with RV or LV, and require an adequate dose and clonal diversity of engrafted cells to reduce the risk of skewed HSCs expansion and stressed hematopoiesis. In the most of LV-based gene therapy trials (ALD, WAS and MLD trials) BM-derived $CD34^+$ cells were harvested as gene transfer target, but recently mobilized PBSC were emerging as a preferential source, especially in adolescents and in adults. The issue of HSC procurement is critical for adult patients, since the minimal target dose ($2–3 \times 10^6$ CD34 cells/kg) poses a challenge for a steady-state BM. In addition, favorable clinical results from gene therapy trial are generally correlated with the dose of transduced cells infused and engrafted. Traditionally the growth factor granulocyte-colony stimulating factor (G-CSF) represents the standard agent to mobilize HSCs for transplantation. In the last years, Plerixafor (AMD3100, Mozobil™), a bicyclam molecule that mobilizes HSCs by selectively and reversibly antagonizing the binding of stromal cell derived factor-1 (SDF-1) to the chemokine CXC receptor-4 (CXCR4) [80], was considered as an alternative mobilization agent. FDA and EMA (European Medical Agency) approved the use of this agent in combination with G-CSF in conditions resulting in poor mobilization with G-CSF alone [81]. To define the optimal mobilization approach for adult patients with β-thalassemia, before the implementation of gene therapy, several mobilization trials were conducted. These studies established that Plerixafor alone and in combination with G-CSF are the optimal mobilization approaches for β-thalassemia gene therapy [82–84]. Ongoing clinical trials of gene therapy for β-thalassemia are mainly using G-CSF + Plerixafor mobilized cells as source of HSCs (Table 1).

A recent study underlined the superior biological and functional features of Plerixafor-mobilized HSCs [85] compared to cells mobilized with G-CSF alone or in combination with Plerixafor. These findings support its use when it is not feasible reaching high target cell dose, or when patients affected by diseases, like SCD, do not tolerate the use of G-CSF as mobilizing agent. In these patients, the inability of erythrocytes to flow in blood vessels and an inflammatory response from chronic hemolysis can induce potential life-threatening crises. In particular, SCD patients can undergo organ ischemia and crises such as acute chest syndrome, stroke, retinal hemorrhage, splenic sequestration, and myocardial infarction. All these complications caused by the disease cannot allow the use of G-CSF. G-CSF was shown to induce a severe pain crisis and acute chest syndrome in these patients [86] and in addition, two serious adverse events were reported in the literature, one in a stem cell donor with SCD who died during G-CSF-induced mobilization [74]. Therefore, current gene therapy clinical trials for SCD are based on BMH, with limitations in procurement of an adequate cell dose. The use of other mobilization agents, different from G-CSF, is desirable. Pilot mobilization trials for SCD patients have been recently opened in the USA and France (clinical trial #NCT03226691,

#NCT02989701, #NCT02193191, #NCT02212535), in which the feasibility of Plerixafor administration for HSC harvest will be tested. Recent data of clinical trial #NCT02193191 [87] in 13 patients support the safety of plerixafor administration at doses of 80, 160 μg/kg, and 240 μg/kg. Plerixafor increased white blood cell counts, absolute neutrophil counts, absolute lymphocyte counts to acceptable levels (1.8–2.8 fold), and allowed the mobilization of high numbers of $CD34^+$ cells for 8 of 13 patients treated at the 3 dose levels.

HGB-206 clinical trial (#NCT02140554) was modified to assess HSC mobilization with plerixafor alone, followed by apheresis and transduction of mobilized cells. Initial results suggest that obtaining adequate doses of $CD34^+$ cells from plerixafor-mobilized PB of patients with SCD may be feasible and safe [79].

4.2. Enrichment and purification of hematopoietic stem cells

In current gene therapy clinical protocols target $CD34^+$ cells are selected by using immunomagnetic beads. The majority of $CD34^+$ cells are short-term progenitor cells with a limited post-transplant lifespan. Only a small fraction of $CD34^+$ cells corresponds to long-term (LT) HSCs. The gene therapy studies conducted in pediatric patients demonstrated that the LT-HSC frequency in BM or mPB $CD34^+$ cells is around 0.01% of the infused dose [88–90] This proportion could be inefficient in a gene therapy setting for disease lacking selective advantage, especially in adult patients where the frequency of HSCs that contribute to long-term engraftment is lower. Indeed, in β-thalassemia and SCD gene therapy trials a large dose of transduced $CD34^+$ cells is required. Isolation of more purified HSCs by FACS sorting, as $CD34^+CD38^-$ cells, could lead to a reduction of transplanted total cell number and LV dose for transduction, although additional enrichment steps would dampen HSC recovery [91]. To date, few clinical trials for hematological malignancies have shown successful transplantation of highly purified HSCs by using CD34 and CD90 cell surface markers [92–94]. The $CD34^+$ $CD90^+$ subpopulation represents 50% of total CD34 + cells. A recent phase 1 clinical study (NCT02963064) will test the safety and tolerability of allogeneic $CD34^+CD90^+$ HSC infused into patients with SCID, pre-conditioned with AMG191 (anti-CD117). Some recent papers postulate that purifying $CD34^+CD38^-$ cells would further enrich for HSCs to be transduced with LV, which importantly are required for long-term clinical benefit after transplantation. The $CD34^+CD38^-$ subpopulation represent 5%–10% of $CD34^+$ cells and is highly enriched for HSCs. Zonari et al., [95] showed a sorting strategy of $CD34^+CD38^-$ cells able to achieve > 90% of HSC activity in < 10% of mobilized peripheral blood (mPB) $CD34^+$ cells, and explore a novel transplantation setting based on uncoupling long-term from short-term hematopoietic reconstitution, and an implemented transduction protocol. Masiuk et al., [96] investigated an immunomagnetic-based method to purify $CD3^+CD38^-$ cells from human BM, mPB and cord blood. They were able to demonstrate the feasibility of this approach, which is available in GMP-grade platform, to reduce the dose of LV without affecting the hematopoietic recovery and long-term engraftment of gene corrected cells in immunodeficient mice. These findings might have a potential impact on the clinical viability of gene therapy for genetic blood diseases affecting a large number of patients.

In the manufacturing process of genetically modified $CD34^+$ cells for clinical applications, a crucial parameter predicting the clinical outcome is the efficiency of vector transduction in the primitive *in vivo* repopulating cells. To date, the lack of available assays testing the still elusive nature of human LT-HSCs imposes the use of surrogate analyses, such as the evaluation by quantitative PCR of proportion of transduced hematopoietic colonies, grown in methylcellulose, or of $CD34^+$ cells shortly expanded *in vitro*. These analyses are used as *bona fide* assay for the stem cell transduction efficiency, even if the results do not represent the amount of transduced cells with long–term engrafting capacity, which is a critical issue impacting the clinical outcome. Transplantation studies of human cells in SCID murine strains allowed estimating the extent of engraftment of SCID repopulating cells (SRCs) [97,98], which represent so far the most primitive human progenitors with hematopoietic reconstitution activity in mice. For obvious reasons this type of assay remains feasible at research level, but cannot be employed as quality test in the manufacturing process of gene therapy drug product.

4.3. Dose of transduced HSCs

In gene therapy, determining the optimal genetically modified cell dose to reach therapeutic effectiveness remains unclear, especially in disease with no selective advantage. The transduced cell dose, as well as the stem cell source, are critical factors that might affect the transplantation outcomes.

A direct correlation of transduced cell dose with clinical outcome is unfeasible on the basis of available results from the clinical trials for β-thalassemia and SCD. Indeed, due to the elusive nature of HSC in humans, the transduced repopulating cell dose is immeasurable by the available preclinical assays. Most of the patients in clinical trials have not been treated with equal cell doses and the VCN in drug products is also variable. No trials using a scalar cell dose have been performed so far. Therefore, the clinical outcome might be influenced by number of different variables, such as the severity of genotype/phenotype, the comorbidities and the secondary modifiers of the pathology, the amount of transduced cells injected, VCN in cell drug product, proportion of engrafting genetically modified long-term HSPCs. Possibly, a multi-parametric analysis should help in order to find any correlation. As a rule of thumb and preliminary indications from clinical trials, patients receiving the highest dose of DP with the highest VCN should have the better outcome.

4.4. Expansion of HSCs

The manufacturing process involving stem cell selection and molecular engineering, by gene transfer or gene editing, is invariably associated with significant cell losses. Therefore, expansion of *ex vivo* modified HSCs would be desirable, to selectively increase stem cell populations endowed with *in vivo* reconstitution potential. A crucial factor that has an impact on the gene transfer efficiency is the low level of *ex vivo* cycling HSCs. Quiescent HSCs are amenable to transduction when they enter cell cycle. Major studies have focused to novel culture conditions, which can lead to HSC proliferation and expansion, while preserving self-renewal and *in vivo* repopulating activity. Different molecules have been tested, including angiopoietin-like proteins, which were shown to induce *ex vivo* expansion of human cells that repopulate immunodeficient mice [99,100]. The aryl hydrocarbon receptor (AhR) antagonist (SR1) and a notch ligand agonist promote *ex vivo* expansion of human $CD34^+$ cells, endowed to engraft immunodeficient mice [101,102]. A recent study showed that a novel small molecule, the pyrimidoindole derivative UM171, expands *ex vivo* cord blood derived $CD34^+$ cells capable of reconstituting human hematopoiesis in immunodeficient mice [103]. This compound acts in independent manner from the AhR, which targets cells with more-limited regenerative potential. Zonari et al. [95] recently demonstrate that, with the use of UM171, the culture time of $CD34^+CD38^-$ cells can be extended beyond 7 days, with evidence of long term hematopoietic stem cell expansion. Overall, although these results indicate some level of HSC expansion, the ideal procedure amenable to provide therapeutic dose of genetically corrected and repopulating HSC is still indefinable.

Since the clinical outcome is expected to correlate not only with the amount of genetically modified HSC, but also with the proportion of cells able to successfully engraft upon transplantation, the use of molecules favoring stem cell engraftment might improve gene therapy protocols. In this context, basic research studies paved the way. A screening of chemicals that induce proliferation of HSCs in zebrafish led to the identification of Prostaglandin E2 (PGE2) as a regulator of HSC homeostasis [104]. PGE2 significantly enhanced engraftment of $CD34^+$

cells after xenotransplantation and showed a stable multilineage engraftment in non-human primate transplant models [105]. These results indicated that PGE2 has evolutionarily conserved functions in regulating HSC numbers and provided sufficient preclinical data to move forward with an FDA-approved phase I clinical trial for its use in cord blood transplantation for subjects with hematological malignancies (clinical trial #NCT00890500).

4.5. Improved transduction methods

In studies of gene transfer, a near-complete transduction of $CD34^+CD38^-$ cells with LV can be achieved by adding PGE2 during a novel short-time cell culture protocol, reducing the negative impact of culture on progenitor cell function [95]. Moreover, the addition of PGE2 increases *in vitro* VCN and/or transduction efficiency of $CD34^+$ cells, at a level depending on the cell source [85]. Recently, Heffener et al., [106] demonstrated that the addition of PGE2 increased lentiviral vector transduction of $CD34^+$ cells approximately 2-fold compared to control transduction methods. Primary $CD34^+$ cells from normal human donors and from patients affected by β-thalassemia or sickle cell disease were efficiently transduced. PGE2 increased also transduction of repopulating human HSPCs in an immune-deficient xenotransplantation mouse model (NSG) without evidence of *in vivo* toxicity, lineage bias, or a *de novo* bias of lentiviral integration sites. These data support the introduction of these improvements in the gene therapy clinical setting and generate new opportunities in the field of gene editing.

In another study, the addition of rapamycin, an inhibitor of the mammalian mTOR pathway, significantly enhanced transduction of HSCs preserving their engraftment potential without alterations in lentiviral integration profile [107]. The authors demonstrated that rapamycin functioned increasing LV post-binding cytoplasmic entry events rather than enhancing autophagy. These data were also confirmed by Petrillo et al. [108]

4.6. Route of administration of HSC

Administration of allogeneic and autologous cells in HSCT is usually given by intravenous injection in patients following myeloablative treatment. The systemic delivery *via* peripheral circulation is an easy procedure aimed to rapid dissemination of transplanted cells, that eventually home and engraft in the BM niche. Unavoidably, a significant proportion of injected cells are lost due to the major filter organs trapping, as lungs and spleen. An alternative route of administration, to overcome cell loss, is represented by the intraosseous injection. This procedure has been successfully utilized in cord blood transplantation, resulting in rapid early engraftment of neutrophils and platelets and a better outcome if compared to i.v. administration [109]. Differently from previous gene therapy studies, in TIGET BTHAL clinical trial (see Section 2 and Table 1) the genetically modified cells are delivered by intraosseous injection, bilaterally in the iliac crests. The choice of this innovative route of administration was supported by data obtained in murine transplantation and imaging studies (MR. Lidonnici, unpublished data) and by the rationale of a direct relocation of HSCs (homing) and their engraftment into the BM niches. Long term follow-up will allow evaluating the real advantage of this procedure in the setting of purified $CD34^+$ cells transplantation.

4.7. Rationale of BM conditioning in gene therapy trials

In the majority of gene therapy trials reduced intensity or non-myeloablative treatment would be preferred to conventional fully myeloablation due to the high risk of non-hematological toxicity. The reduced conditioning regimen can lead to mixed chimerism, which can provide clinical benefit only if the gene-corrected cells are endowed with a selective advantage, as observed in clinical trials for immunodeficiencies [59,88,110–112].

Partially myeloablative conditioning with busulfan was used in the context of gene therapy trial for patients affected by WAS with RV expressing Wasp from viral LTR promoter. Stable engraftment of transduced cells was achieved at multilineage level in both BM and PB, with a selective advantage conferred by the transgene to the lymphoid cells [63]. In a LV-based trials for WAS in pediatric and adult patients [88,111,112], a reduced intensity conditioning led to the presence of transduced HSCs in the 20–50% of stem cell compartment of the BM, with a higher proportion of transduced cells in the lymphoid subset. Also in this case, the reduced intensity conditioning led to a clinical benefit due to the selective advantage conferred by the transgene to the lymphoid cells. Moreover, the first report of successful GT in an adult with severe WAS was recently reported [112]. In gene therapy trials for metachromatic leukodystrophy [113] and CGD [114], in which high levels of engraftment of gene corrected HSCs are required and no selective advantage is documented, the choice fell on fully myeloablative conditioning. Noteworthy, in the gene therapy trial for β-thalassemia sponsored by MSKCC, low dose of busulfan resulted in insufficient engraftment of gene marked cells and lack of significant clinical benefit [71]. The BM of thalassemic patients contains a number of erythroid precursors five to six-fold higher than that of healthy controls [115]. The presence of expanded erythroid precursors in the BM and the reduced myeloablative treatment, unable to eradicate the erythron growth, could explain low engraftment of genetically modified HSCs in these patients. Thus, based on these early results, the subsequent trials for hemoglobinopathies adopted full myeloablative treatment in order to make adequate space in the BM niches and achieve a high level of engraftment of corrected HSCs.

For genetic disorders, it is mandatory to develop optimal conditioning strategies with minimal toxicity but sufficient myeloablative efficacy to achieve therapeutic levels of genetically corrected HSCs. New advanced treatments to myeloablate BM niches with biological agents, without using chemicals, like anti-cKit or anti-CD45-SAP antibodies are being exploited [116–118].

Studies performed in murine models demonstrated the efficacy of using monoclonal antibodies directed against cell surface antigens expressed by all blood cells, such as CD45, or by subpopulations enriched for HSC, such as c-KIT, to deplete resident HSC [119,120]. Moreover, anti-CD45 antibodies (YTH24.5 and YTH 54.12) have already been tested successfully in combination with immune suppressive drugs in a clinical trial of allogeneic HSCT in patients with pre-existing organ toxicity or DNA repair defects [121]. Monoclonal antibody-based conditioning was well tolerated and achieved curative engraftment.

Recently, a novel approach has been developed employing anti-CD45–SAP, a hematopoietic-cell-specific immunotoxin consisting of saporin (SAP) conjugated to a CD45-targeting antibody. Saporin, a ricin family toxin able to halt protein synthesis, coupled to an anti-CD45 antibody was administered to wild-type mice as a conditioning regimen before HSCT. The treatment was capable of depleting HSCs nearly as total body irradiation (TBI). CD45-SAP permitted long-term stable donor chimerism, with less toxicity, more rapid immune recovery, better preservation of BM and thymus architecture than conventional methods [122].

As an alternative to chemotherapy or radiation, a new immunotherapy strategy that clears out host HSCs from the BM in preparation for a transplant was described in Chhabra et al. [123]. The authors demonstrated that blockade of the surface antigen CD47 allows phagocytic myeloid cells to engulf host HSCs displaced by anti-c-kit antibody (ACK2). CD47 blockade enhances the potency of ACK2 for transplant conditioning. This combined treatment led to depletion of 99% of HSCs from the BM of immunocompetent mice, to improved engraftment of donor HSCs, with fewer toxic effects for recipient mice. Clinical trials using these approaches are currently running in patients with advanced solid and hematologic cancers (clinical trial #NCT02216409 and NCT02367196). Recently, a phase 1 study has

been opened to assess the safety and tolerability of HSC allografts infused into patients affected by SCID, treated before transplantation with AMG 191, an antibody that targets CD117 (c-kit) present on endogenous HSC (clinical trial #NCT02963064). Interestingly, in the model of Fanconi anemia mice, the use of anti c-kit alone was shown to be sufficient for donor HSC engraftment [124].

All these novel conditioning approaches will enable autologous HSC transplants to be more effective with reduced toxicity in diseases such as hemoglobinopathies.

4.8. The age factor

In the design of gene therapy studies the inclusion of patients with different age needs to be carefully evaluated and justified, and the outcome might be affected by age-related response to treatment. Some clues relevant in the context of autologous transplantation can be inferred from the clinical experience in the allogeneic setting.

This is particularly evident from the results of allogeneic HSCT in β-thalassemia, with better results in younger patients *vs* the adults. Furthermore, age is an important factor influencing the function of both HSCs and microenvironment, since hemoglobinopathies are progressive diseases. The altered status of the bone marrow due to ineffective erythropoiesis, iron overload, ROS accumulation and a general hypermetabolic state might affect long-term HSC functions and the support by the niche.

Clinical HSCT faces two obstacles that can affect the success of this treatment: insufficient numbers of transplantable stem cells and low engraftment efficiency. These limitations are intensified by patients' advanced age [125] [126]. The National Marrow Donor Program has reported that donor age is the only factor significantly associated with overall survival rate of transplanted recipients: the younger the donor, the better the long-term outcome [127]. Blood cells are produced regularly from the BM as a result of a homeostatic mechanism that regulates cell production and differentiation within the hematopoietic system. During the aging process HSCs are subjected to exhaustion, both quantitatively and qualitatively. Even though HSCs increase in number with age [128,129], they lose their repopulating abilities upon transplantation [130]. In addition, a decrease in HSC function was negatively correlated with lifespan [131]. From these studies, it is clear that enhanced *in vivo* proliferation can lead to exhaustion of the HSC pool and reduction of its quality, limiting self-renewal capacity, homing and engraftment potentials. Major phenotypes are associated with hematopoietic aging and recent studies investigate cell-intrinsic and cell-extrinsic mechanisms of HSC aging (reviewed in [132]).

The disease prognosis and stage contribute to the inferior outcome of allogeneic HSCT in older patients compared with younger patients. In gene therapy trials of progressive diseases, the severity of the pathology correlates with the age and influences the clinical benefit [88,89]. With the exceptions of MLD and ALD, in all other diseases the HSC content and relationship with the stroma are affected by the pathology [133–140]. The results from TIGET BTHAL clinical trial, where different age groups of patients are included, from 3 to > 18 years old, will be instrumental for studying the age factor in autologous transplantation of genetically modified HSCs.

4.9. The role of BM microenvironment

Since the performance status of patients affected by β-hemoglobinopathies is increasingly deteriorated, the BM microenvironment might affect the HSC features. The non-hematopoietic component of the BM microenvironment supports the engraftment of HSCs. Peculiar features of β-thalassemia, as anemia, extramedullary hematopoiesis, iron accumulation and bone deformities due to marrow expansion, can alter the BM microenvironment in patients. Preliminary data showed the impact of an altered microenvironment on HSC behavior and function in thalassemia intermedia murine model [141]. Further investigation in the human context will be instructive to understand the basis for reconstituting hematopoiesis and effective erythropoiesis in a chronically stressed environment. Interestingly, SCD patients show BM alterations, as erythroid hyperplasia, with extensive marrow erythrophagocytosis, an abnormally low reticulocyte response, presence of hemoglobin S polymers in reticulocytes, sickling of nucleated erythroblasts [142,143]. These features associated with endothelial activation and a general reactive milieu might affect the homeostasis and egress from the BM of $CD34^+$ cells [144]. All these aspects must be taken into account when designing gene therapy trials.

Several endocrine factors, such as erythropoietin (Epo), parathyroid hormone (PTH) and estrogens, which are altered in patients, are known to regulate HSC maintenance, self-renewal and differentiation by modulating the mesenchymal stromal function and osteoblastic activity [145–148]. Whereas it is speculative to consider that continuous and excessive elevation of endogenous Epo level or reduction of PTH may lead to alterations of the bone microenvironment, it is well documented that iron accumulation can affect the normal hematopoiesis through an alteration of BM niche in a disease context. Iron is usually involved in many physiological cellular functions such as cell cycle, DNA replication, metabolism and DNA synthesis/repair and processes that occur in mitochondria [149]. Recent data in patients affected by myelodysplastic syndromes (MDS) and leukemia support the hypothesis that iron overload through reactive oxygen species (ROS) production may have a negative effect on hematopoiesis and the hematological niche. These diseases share some feature with β-hemoglobinopathies, as iron overload, caused by ineffective erythropoiesis, increased gastrointestinal iron intake and transfusion dependency.

Recent studies suggest that ROS are implicated in HSC state and function. Ludin and colleagues showed how ROS levels are essential to maintain the self-renewal of stem cells [150]. Elevation in ROS content appears to drive HSC out of quiescence, to reduce self-renewal capacity and to promote migration to the vascular side of the niche, where the microenvironment induces HSCs to proliferate and differentiate. Several environmental factors [151] and hypoxic conditions [152] can contribute to maintain low ROS levels. However, extremely low ROS levels can prime HSCs to differentiate leading to impaired repopulation capacity [153]. On the other hand, high ROS levels can induce stem cell differentiation towards myeloid lineage [154,155]. Stress conditions, such as iron overload, through ROS production could impair self-renewal capacity of HSCs [156]. Moreover, iron overload can damage BM stroma, liver, kidney and indirectly influences the engraftment of normal HSCs by reducing expression of molecules, such as CXCL12, VCAM-1, Kit-ligand, Epo and thrombopoietin [157].

Therefore, in the setting of autologous HSC transplantation, as in gene therapy, the two fundamental cellular components of the hematopoietic system- HSC and its niche- might be both affected by aging and/or by disease related stress factors inducing premature senescence.

5. Genome-editing technologies: gene correction *versus* gene addition

Genome-editing technologies offer an innovative approach for treating β-hemoglobinopathies (Fig. 1A and B). In the last decades, several nucleases were developed for genetic engineering: zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeat (CRISPR)-associated nuclease Cas9 (Fig. 1A). Clinical gene-editing trials to treat diseases such as HIV, leukemia, hemophilia B, mucopolysaccharidosis I, both by *ex vivo* and *in vivo* methods, have been started in the last 2 years [158].

The CRISPR/Cas9 system is the most novel approach, using a single or multiple short guide RNAs (gRNA) with 20 bp complementary to DNA sequence targeted [159]. The nucleases create site specific double strand breaks (DBSs) which can be repaired by two mechanisms: non-homologous end joining (NHEJ), with direct fusion of the cleaved ends,

and homologous directed repair (HDR) which needs an exogenous template [160], usually delivered *via* single-stranded oligodeoxynucleotides or viral vectors as integrase defective LV or AAV [161] for gene correction with insertion.

In the context of β-hemoglobinopathies, different gene editing strategies have been successful, including induction of endogenous fetal γ-globin [162], correction of β-globin gene mutation [163–165], or a combined approach [166,167]. Recently, inactivation by genome editing of BCL11A, a validated HbF repressor and therapeutic target for β-hemoglobin disorders has been proposed [168–171]. Common genetic variants at BCL11A locus were found to be associated with HbF level [172] and suppression of BCL11A in the erythroid lineage increased the γ-globin gene expression [172–174].

It has been observed that ubiquitous BCL11A knockdown impaired engraftment of both human and mouse repopulating HSCs [175,176]. These data suggest that global targeting of BCL11A in stem and progenitor cells, such as human $CD34^+$ cells, is inappropriate. Therapeutic strategies that emphasize disrupting BCL11A selectively in erythroid cells need to be considered.

To develop an effective and sustainable cell therapy for SCD, Chang et al. [177] investigated the feasibility of targeted disruption of the BCL11A gene, either within exon 2 or at the GATAA motif of the intronic erythroid-specific enhancer, using ZFNs in BM $CD34^+$ HSCs. The authors showed that BCL11A gene disruption impaired erythroid enucleation, whereas bi-allelic disruption of the GATAA motif in the erythroid enhancer of BCL11A did not affect enucleation and HSCs function.

The erythroid-specific BCL11A knockdown, by gene transfer of LV carrying shRNA, demonstrated that its expression is required to maintain the silencing of HbF expression in erythroid cells [176] without toxicity on HSCs. Moreover, unlike gene editing, the use of LVs has the advantage that several factors important for clinical translation such as safety, efficacy, and high transduction efficiencies were already addressed from a decade of clinical studies. This study laid the foundation for a recently approved clinical trial for treating SCD (clinical trial #NCT03282656).

Recently, using ZFNs or CRISPR/Cas9 nucleases combined with single-strand oligodeoxynucleotide donors or integrase-defective LVs carrying the donor templates, site-specific correction of the sickle mutation in HSCs allowed permanent production of normal red blood cells [164,165,178]. However, gene targeting in primary immunodeficiencies showed that a high gene targeting efficiency in HSCs by homologous recombination at disease-causing loci is difficult to obtain [179]. HDR is mainly active during the S/G2 phase, whereas NHEJ is active throughout the entire cell cycle and therefore HSCs are less permissive to genome engineering.

Recently a new approach reached efficient homologous-recombination-mediated editing frequencies at the HBB locus in $CD34^+$ HSCs by using Cas9 ribonucleoprotein combined with rAAV6 homologous donor delivery [180]. They demonstrated efficient correction of Glu6Val mutation in HSCs derived from several patients with SCD and persistence of modified human cells in secondary transplants of immunodeficient mice.

The generation of hereditary persistence of fetal hemoglobin (HPFH) mutations in HSCs by genome-editing system could be an alternative therapeutic strategy to increase HbF levels in both β-thalassemic and SCD patients. Wienert B et al. demonstrated that the introduction of the HPFH-175 T > C point mutation is associated with elevated fetal γ-globin expression in erythroid cell lines through *de novo* recruitment of the activator TAL1 [181]. Recently, another study used the CRISPR/Cas9 system to mutate a 13-nt sequence in the promoters of the HBG1 and HBG2 genes *via* microhomology-mediated end-joining (MMEJ), leading to HbF increase to potentially therapeutic levels [182].

Future studies are required to assess potential toxicity of genome editing in human HSCs and to minimize potentially unsafe off-target mutations before clinical translation [183]. Recently several researches try to develop unbiased method to assess off target mutagenesis [184–186] [187]. Some other important issues regarding cytotoxicity must be addressed, as high vector doses employed in HSCs and the occasionally unfavorable ratio of HDR to NHEJ, as HDR-based correction of mutation may restore expression of a functional β-globin, whereas NHEJ-mediated disruption of the same locus could generate a β-thalassemia phenotype.

A completely different and novel approach has been very recently reported by directly reducing expression of α-globin, since the excess of α-globin chains has an important impact on the pathophysiology of β-thalassemia. The authors used CRISPR/Cas9 genome editing of primary human $CD34^+$ cells to mimic a natural mutation, which consists in the deletion of the MCS-R2 α-globin enhancer causing α-thalassemia [188]. In this case, the rationale of altering α-globin production is based on the potential benefit associated to the decrease of uncoupled free α-chains in β-thalassemia. Edited $CD34^+$ cells obtained from patients affected by β-thalassemia were able to differentiate into erythroid cells, with a reduction in α-globin expression and a correction of the α/β globin chains imbalance. Moreover, the edited $CD34^+$ cells showed a long-term repopulating capacity in immunodeficient mice, demonstrating that the treatment does not compromise the *in vivo* HSC engraftment in the hematological chimera model.

Overall, results from gene editing methods to correct β-hemoglobinopathies are promising and the potential clinical translation needs to be evaluated in preclinical genotoxicity and safety studies, in the best available models. In addition, preclinical efficacy studies should allow establishing advantages or disadvantages in terms of potential therapeutic benefit *versus* the current gene therapy approaches.

6. Concluding remarks

Although several barriers to gene therapy appear to have been overcome, some obstacles remain to be solved. A number of years of clinical application of gene transfer techniques have highlighted some benefits as well as the current limitations of *ex vivo* gene therapy for hemoglobinopathies. Using gene editing instead of gene addition does not change some fundamental, still-open issues about autologous cell transplantation in SCD and β-thalassemia, such as stem cell sources and preservation of HSC functions upon gene modifications. Introducing small or large deletions in the HSC genome by NHEJ is relatively efficient, and not particularly cumbersome. Gene deletions are not an obvious target for hemoglobinopathies, nevertheless the disruption of molecular repressors of fetal globin genes and the generation of mutations reproducing the HPFH condition are both promising strategies. Gene correction by HR-mediated DNA repair, that requires a different molecular strategy for each mutation, is complex and is less efficient than NHEJ in HSCs. The consequences of introducing double-stranded DNA breaks in somatic stem cells are far from being understood. Therefore, currently gene editing cannot be considered safer than viral-mediated gene addition.

References

[1] Y.W. Kan, Molecular biology of hemoglobin: its application to sickle cell anemia and thalassemia, Schweiz. Med. Wochenschr. Suppl. 43 (1991) 51–54.
[2] B. Modell, M. Darlison, Global epidemiology of haemoglobin disorders and derived service indicators, Bull. World Health Organ. 86 (2008) 480–487.
[3] F.B. Piel, A.P. Patil, R.E. Howes, O.A. Nyangiri, P.W. Gething, M. Dewi, W.H. Temperley, T.N. Williams, D.J. Weatherall, S.I. Hay, Global epidemiology of sickle haemoglobin in neonates: a contemporary geostatistical model-based map and population estimates, Lancet 381 (2013) 142–151.
[4] National Heart, Lung, Blood Institute, Evidence-based management of sickle cell disease, Expert Panel Report, 2014 https://catalog.nhlbi.nih.gov.
[5] V.M. Ingram, Abnormal human haemoglobins. III. The chemical difference between normal and sickle cell haemoglobins, Biochim. Biophys. Acta 36 (1959) 402–411.
[6] K.A.P. Bridges, A. H, Anemias and Other Red Cell Disorders, 1st Edition, McGraw-Hill Education/Medical, 2008.

[7] M.H. Steinberg, S.H. Embury, Alpha-thalassemia in blacks: genetic and clinical aspects and interactions with the sickle hemoglobin gene, Blood 68 (1986) 985–990.
[8] O.S. Platt, Hydroxyurea for the treatment of sickle cell anemia, N. Engl. J. Med. 358 (2008) 1362–1369.
[9] F.A. Ferrone, Sickle cell disease: its molecular mechanism and the one drug that treats it, Int. J. Biol. Macromol. 93 (2016) 1168–1173.
[10] C. Madigan, P. Malik, Pathophysiology and therapy for haemoglobinopathies. Part I: sickle cell disease, Expert Rev. Mol. Med. 8 (2006) 1–23.
[11] E. Vichinsky, E. Butensky, E. Fung, M. Hudes, E. Theil, L. Ferrell, R. Williams, L. Louie, P.D. Lee, P. Harmatz, Comparison of organ dysfunction in transfused patients with SCD or beta thalassemia, Am. J. Hematol. 80 (2005) 70–74.
[12] D.J. Weatherall, J.B. Clegg, Inherited haemoglobin disorders: an increasing global health problem, Bull. World Health Organ. 79 (2001) 704–712.
[13] A.T. Taher, D.J. Weatherall, M.D. Cappellini, Thalassaemia, Lancet (2017).
[14] E. Angelucci, S. Matthes-Martin, D. Baronciani, F. Bernaudin, S. Bonanomi, M.D. Cappellini, J.H. Dalle, P. Di Bartolomeo, C.D. de Heredia, R. Dickerhoff, C. Giardini, E. Gluckman, A.A. Hussein, N. Kamani, M. Minkov, F. Locatelli, V. Rocha, P. Sedlacek, F. Smiers, I. Thuret, I. Yaniv, M. Cavazzana, C. Peters, E.I. Error, E.P.W. Parties, Hematopoietic stem cell transplantation in thalassemia major and sickle cell disease: indications and management recommendations from an international expert panel, Haematologica 99 (2014) 811–820.
[15] D. Baronciani, E. Angelucci, U. Potschger, J. Gaziev, A. Yesilipek, M. Zecca, M.G. Orofino, C. Giardini, A. Al-Ahmari, S. Marktel, J. de la Fuente, A. Ghavamzadeh, A.A. Hussein, C. Targhetta, F. Pilo, F. Locatelli, G. Dini, P. Bader, C. Peters, Hemopoietic stem cell transplantation in thalassemia: a report from the European Society for Blood and Bone Marrow Transplantation Hemoglobinopathy Registry, 2000–2010, Bone Marrow Transplant. 51 (2016) 536–541.
[16] E. Gluckman, B. Cappelli, F. Bernaudin, M. Labopin, F. Volt, J. Carreras, B. Pinto Simoes, A. Ferster, S. Dupont, J. de la Fuente, J.H. Dalle, M. Zecca, M.C. Walters, L. Krishnamurti, M. Bhatia, K. Leung, G. Yanik, J. Kurtzberg, N. Dhedin, M. Kuentz, G. Michel, J. Apperley, P. Lutz, B. Neven, Y. Bertrand, J.P. Vannier, M. Ayas, M. Cavazzana, S. Matthes-Martin, V. Rocha, H. Elayoubi, C. Kenzey, P. Bader, F. Locatelli, A. Ruggeri, M. Eapen, t.P.W.P.o.t.E.S.f.B. Eurocord, T. Marrow, B, the Center for International, R. Marrow Transplant, Sickle cell disease: an international survey of results of HLA-identical sibling hematopoietic stem cell transplantation, Blood 129 (2017) 1548–1556.
[17] R. H, S. Philipsen, Evolution of hemoglobin loci and their regulatory elements, Blood Cells Mol. Dis. S1079–9796 (1017) (2017) (30148-30141).
[18] C. Paszty, C.M. Brion, E. Manci, H.E. Witkowska, M.E. Stevens, N. Mohandas, E.M. Rubin, Transgenic knockout mice with exclusively human sickle hemoglobin and sickle cell disease, Science 278 (1997) 876–878.
[19] M. Trudel, N. Saadane, M.C. Garel, J. Bardakdjian-Michau, Y. Blouquit, J.L. Guerquin-Kern, P. Rouyer-Fessard, D. Vidaud, A. Pachnis, P.H. Romeo, et al., Towards a transgenic mouse model of sickle cell disease: hemoglobin SAD, EMBO J. 10 (1991) 3157–3165.
[20] L.C. Skow, B.A. Burkhart, F.M. Johnson, R.A. Popp, D.M. Popp, S.Z. Goldberg, W.F. Anderson, L.B. Barnett, S.E. Lewis, A mouse model for beta-thalassemia, Cell 34 (1983) 1043–1052.
[21] B. Yang, S. Kirby, J. Lewis, P.J. Detloff, N. Maeda, O. Smithies, A mouse model for beta 0-thalassemia, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 11608–11612.
[22] R.D. Cone, A. Weber-Benarous, D. Baorto, R.C. Mulligan, Regulated expression of a complete human beta-globin gene encoded by a transmissible retrovirus vector, Mol. Cell. Biol. 7 (1987) 887–897.
[23] E.A. Dzierzak, T. Papayannopoulou, R.C. Mulligan, Lineage-specific expression of a human beta-globin gene in murine bone marrow transplant recipients reconstituted with retrovirus-transduced stem cells, Nature 331 (1988) 35–41.
[24] U. Novak, E.A. Harris, W. Forrester, M. Groudine, R. Gelinas, High-level beta-globin expression after retroviral transfer of locus activation region-containing human beta-globin gene derivatives into murine erythroleukemia cells, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 3386–3390.
[25] J.C. Chang, D. Liu, Y.W. Kan, A 36-base-pair core sequence of locus control region enhances retrovirally transferred human beta-globin gene expression, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 3107–3110.
[26] I. Plavec, T. Papayannopoulou, C. Maury, F. Meyer, A human beta-globin gene fused to the human beta-globin locus control region is expressed at high levels in erythroid cells of mice engrafted with retrovirus-transduced hematopoietic stem cells, Blood 81 (1993) 1384–1392.
[27] L. Naldini, U. Blomer, P. Gallay, D. Ory, R. Mulligan, F.H. Gage, I.M. Verma, D. Trono, In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector, Science 272 (1996) 263–267.
[28] C. May, S. Rivella, J. Callegari, G. Heller, K.M. Gaensler, L. Luzzatto, M. Sadelain, Therapeutic haemoglobin synthesis in beta-thalassaemic mice expressing lentivirus-encoded human beta-globin, Nature 406 (2000) 82–86.
[29] S. Imren, E. Payen, K.A. Westerman, R. Pawliuk, M.E. Fabry, C.J. Eaves, B. Cavilla, L.D. Wadsworth, Y. Beuzard, E.E. Bouhassira, R. Russell, I.M. London, R.L. Nagel, P. Leboulch, R.K. Humphries, Permanent and panerythroid correction of murine beta thalassemia by multiple lentiviral integration in hematopoietic stem cells, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 14380–14385.
[30] S. Rivella, C. May, A. Chadburn, I. Riviere, M. Sadelain, A novel murine model of Cooley anemia and its rescue by lentiviral-mediated human beta-globin gene transfer, Blood 101 (2003) 2932–2939.
[31] H. Hanawa, P.W. Hargrove, S. Kepes, D.K. Srivastava, A.W. Nienhuis, D.A. Persons, Extended beta-globin locus control region elements promote consistent therapeutic expression of a gamma-globin lentiviral vector in murine beta-thalassemia, Blood 104 (2004) 2281–2290.
[32] A. Miccio, R. Cesari, F. Lotti, C. Rossi, F. Sanvito, M. Ponzoni, S.J. Routledge, C.M. Chow, M.N. Antoniou, G. Ferrari, In vivo selection of genetically modified erythroblastic progenitors leads to long-term correction of beta-thalassemia, Proc. Natl. Acad. Sci. U. S. A. 105 (2008) 10547–10552.
[33] L. Breda, C. Casu, S. Gardenghi, N. Bianchi, L. Cartegni, M. Narla, K. Yazdanbakhsh, M. Musso, D. Manwani, J. Little, L.B. Gardner, D.A. Kleinert, E. Prus, E. Fibach, R.W. Grady, P.J. Giardina, R. Gambari, S. Rivella, Therapeutic hemoglobin levels after gene transfer in beta-thalassemia mice and in hematopoietic cells of beta-thalassemia and sickle cells disease patients, PLoS One 7 (2012) e32345.
[34] A. Miccio, V. Poletti, F. Tiboni, C. Rossi, A. Antonelli, F. Mavilio, G. Ferrari, The GATA1-HS2 enhancer allows persistent and position-independent expression of a beta-globin transgene, PLoS One 6 (2011) e27955.
[35] L. Lisowski, M. Sadelain, Locus control region elements HS1 and HS4 enhance the therapeutic efficacy of globin gene transfer in beta-thalassemic mice, Blood 110 (2007) 4175–4178.
[36] P.I. Arumugam, J. Scholes, N. Perelman, P. Xia, J.K. Yee, P. Malik, Improved human beta-globin expression from self-inactivating lentiviral vectors carrying the chicken hypersensitive site-4 (cHS4) insulator element, Mol. Ther. 15 (2007) 1863–1871.
[37] M. Kumar, B. Keller, N. Makalou, R.E. Sutton, Systematic determination of the packaging limit of lentiviral vectors, Hum. Gene Ther. 12 (2001) 1893–1905.
[38] M. Andreani, M. Testi, J. Gaziev, R. Condello, A. Bontadini, P.L. Tazzari, F. Ricci, L. De Felice, F. Agostini, D. Fraboni, G. Ferrari, M. Battarra, M. Troiano, P. Sodani, G. Lucarelli, Quantitatively different red cell/nucleated cell chimerism in patients with long-term, persistent hematopoietic mixed chimerism after bone marrow transplantation for thalassemia major or sickle cell disease, Haematologica 96 (2011) 128–133.
[39] G. Puthenveetil, P. Malik, Gene therapy for hemoglobinopathies: are we there yet? Curr. Hematol. Rep. 3 (2004) 298–305.
[40] E.A. Roselli, R. Mezzadra, M.C. Frittoli, G. Maruggi, E. Biral, F. Mavilio, F. Mastropietro, A. Amato, G. Tonon, C. Refaldi, M.D. Cappellini, M. Andreani, G. Lucarelli, M.G. Roncarolo, S. Marktel, G. Ferrari, Correction of beta-thalassemia major by gene transfer in haematopoietic progenitors of pediatric patients, EMBO Mol. Med. 2 (2010) 315–328.
[41] M.D. Milsom, D.A. Williams, Gaining the hard yard: pre-clinical evaluation of lentiviral-mediated gene therapy for the treatment of beta-thalassemia, EMBO Mol. Med. 2 (2010) 291–293.
[42] S.L. McCune, M.P. Reilly, M.J. Chomo, T. Asakura, T.M. Townes, Recombinant human hemoglobins designed for gene therapy of sickle cell disease, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 9852–9856.
[43] R. Pawliuk, K.A. Westerman, M.E. Fabry, E. Payen, R. Tighe, E.E. Bouhassira, S.A. Acharya, J. Ellis, I.M. London, C.J. Eaves, R.K. Humphries, Y. Beuzard, R.L. Nagel, P. Leboulch, Correction of sickle cell disease in transgenic mouse models by gene therapy, Science 294 (2001) 2368–2371.
[44] D.N. Levasseur, T.M. Ryan, K.M. Pawlik, T.M. Townes, Correction of a mouse model of sickle cell disease: lentiviral/antisickling beta-globin gene transduction of unmobilized, purified hematopoietic stem cells, Blood 102 (2003) 4312–4319.
[45] Z. Romero, F. Urbinati, S. Geiger, A.R. Cooper, J. Wherley, M.L. Kaufman, R.P. Hollis, R.R. de Assin, S. Senadheera, A. Sahagian, X. Jin, A. Gellis, X. Wang, D. Gjertson, S. Deoliveira, P. Kempert, S. Shupien, H. Abdel-Azim, M.C. Walters, H.J. Meiselman, R.B. Wenby, T. Gruber, V. Marder, T.D. Coates, D.B. Kohn, Beta-globin gene transfer to human bone marrow for sickle cell disease, J. Clin. Invest. 123 (2013) 3317–3330.
[46] T.I. Pestina, P.W. Hargrove, D. Jay, J.T. Gray, K.M. Boyd, D.A. Persons, Correction of murine sickle cell disease using gamma-globin lentiviral vectors to mediate high-level expression of fetal hemoglobin, Mol. Ther. 17 (2009) 245–252.
[47] D.A. Persons, P.W. Hargrove, E.R. Allay, H. Hanawa, A.W. Nienhuis, The degree of phenotypic correction of murine beta-thalassemia intermedia following lentiviral-mediated transfer of a human gamma-globin gene is influenced by chromosomal position effects and vector copy number, Blood 101 (2003) 2175–2183.
[48] A. Perumbeti, T. Higashimoto, F. Urbinati, R. Franco, H.J. Meiselman, D. Witte, P. Malik, A novel human gamma-globin gene vector for genetic correction of sickle cell anemia in a humanized sickle mouse model: critical determinants for successful correction, Blood 114 (2009) 1174–1185.
[49] M. White, R. Whittaker, C. Gandara, E.A. Stoll, A guide to approaching regulatory considerations for lentiviral-mediated gene therapies, Hum. Gene Ther. Methods 28 (2017) 163–176.
[50] D.W. Emery, E. Yannaki, J. Tubb, T. Nishino, Q. Li, G. Stamatoyannopoulos, Development of virus vectors for gene therapy of beta chain hemoglobinopathies: flanking with a chromatin insulator reduces gamma-globin gene silencing in vivo, Blood 100 (2002) 2012–2019.
[51] A. Ramezani, T.S. Hawley, R.G. Hawley, Combinatorial incorporation of enhancer-blocking components of the chicken beta-globin 5'HS4 and human T-cell receptor alpha/delta BEAD-1 insulators in self-inactivating retroviral vectors reduces their genotoxic potential, Stem Cells 26 (2008) 3257–3266.
[52] R. Desprat, E.E. Bouhassira, Gene specificity of suppression of transgene-mediated insertional transcriptional activation by the chicken HS4 insulator, PLoS One 4 (2009) e5956.
[53] M. Cavazzana-Calvo, E. Payen, O. Negre, G. Wang, K. Hehir, F. Fusil, J. Down, M. Denaro, T. Brady, K. Westerman, R. Cavallesco, B. Gillet-Legrand, L. Caccavelli, R. Sgarra, L. Maouche-Chretien, F. Bernaudin, R. Girot, R. Dorazio, G.J. Mulder, A. Polack, A. Bank, J. Soulier, J. Larghero, N. Kabbara, B. Dalle, B. Gourmel, G. Socie, S. Chretien, N. Cartier, P. Aubourg, A. Fischer, K. Cornetta, F. Galacteros, Y. Beuzard, E. Gluckman, F. Bushman, S. Hacein-Bey-Abina, P. Leboulch, Transfusion independence and HMGA2 activation after gene therapy of human

beta-thalassaemia, Nature 467 (2010) 318–322.
[54] A. Moiani, Y. Paleari, D. Sartori, R. Mezzadra, A. Miccio, C. Cattoglio, F. Cocchiarella, M.R. Lidonnici, G. Ferrari, F. Mavilio, Lentiviral vector integration in the human genome induces alternative splicing and generates aberrant transcripts, J. Clin. Invest. 122 (2012) 1653–1666.
[55] O. Negre, C. Bartholomae, Y. Beuzard, M. Cavazzana, L. Christiansen, C. Courne, A. Deichmann, M. Denaro, E. de Dreuzy, M. Finer, R. Fronza, B. Gillet-Legrand, C. Joubert, R. Kutner, P. Leboulch, L. Maouche, A. Paulard, F.J. Pierciey, M. Rothe, B. Ryu, M. Schmidt, C. von Kalle, E. Payen, G. Veres, Preclinical evaluation of efficacy and safety of an improved lentiviral vector for the treatment of beta-thalassemia and sickle cell disease, Curr. Gene Ther. 15 (2015) 64–81.
[56] M.R. Lidonnici, F. Salvatori, F. Tiboni, G. Mandelli, Y. Paleari, A. Aprile, F. Sanvito, P. Cristofori, F. Benedicenti, A. Calabria, E. Montini, G. Ferrari, High dose of transduced HPSCs provides safe long term correction of beta-thalassemia, The European Society for Gene and Cell Therapy and the Spanish Society for Gene and Cell Therapy Collaborative Congress 2013, Human Gene Therapy, Palacio Municipal de Congresos Madrid, Spain, 2013, pp. A109–110.
[57] F. Urbinati, J. Wherley, S. Geiger, B.C. Fernandez, M.L. Kaufman, A. Cooper, Z. Romero, F. Marchioni, L. Reeves, E. Read, B. Nowicki, E. Grassman, S. Viswanathan, X. Wang, R.P. Hollis, D.B. Kohn, Preclinical studies for a phase 1 clinical trial of autologous hematopoietic stem cell gene therapy for sickle cell disease, Cytotherapy 19 (2017) 1096–1112.
[58] S. Ghosh, H.B. Gaspar, Gene therapy approaches to immunodeficiency, Hematol. Oncol. Clin. North Am. 31 (2017) 823–834.
[59] C. Booth, H.B. Gaspar, A.J. Thrasher, Treating immunodeficiency through HSC gene therapy, Trends Mol. Med. 22 (2016) 317–327.
[60] S. Hacein-Bey-Abina, A. Garrigue, G.P. Wang, J. Soulier, A. Lim, E. Morillon, E. Clappier, L. Caccavelli, E. Delabesse, K. Beldjord, V. Asnafi, E. MacIntyre, L. Dal Cortivo, I. Radford, N. Brousse, F. Sigaux, D. Moshous, J. Hauer, A. Borkhardt, B.H. Belohradsky, U. Wintergerst, M.C. Velez, L. Leiva, R. Sorensen, N. Wulffraat, S. Blanche, F.D. Bushman, A. Fischer, M. Cavazzana-Calvo, Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1, J. Clin. Invest. 118 (2008) 3132–3142.
[61] S.J. Howe, M.R. Mansour, K. Schwarzwaelder, C. Bartholomae, M. Hubank, H. Kempski, M.H. Brugman, K. Pike-Overzet, S.J. Chatters, D. de Ridder, K.C. Gilmour, S. Adams, S.I. Thornhill, K.L. Parsley, F.J. Staal, R.E. Gale, D.C. Linch, J. Bayford, L. Brown, M. Quaye, C. Kinnon, P. Ancliff, D.K. Webb, M. Schmidt, C. von Kalle, H.B. Gaspar, A.J. Thrasher, Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients, J. Clin. Invest. 118 (2008) 3143–3150.
[62] S. Stein, M.G. Ott, S. Schultze-Strasser, A. Jauch, B. Burwinkel, A. Kinner, M. Schmidt, A. Kramer, J. Schwable, H. Glimm, U. Koehl, C. Preiss, C. Ball, H. Martin, G. Gohring, K. Schwarzwaelder, W.K. Hofmann, K. Karakaya, S. Tchatchou, R. Yang, P. Reinecke, K. Kuhlcke, B. Schlegelberger, A.J. Thrasher, D. Hoelzer, R. Seger, C. von Kalle, M. Grez, Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease, Nat. Med. 16 (2010) 198–204.
[63] C.J. Braun, K. Boztug, A. Paruzynski, M. Witzel, A. Schwarzer, M. Rothe, U. Modlich, R. Beier, G. Gohring, D. Steinemann, R. Fronza, C.R. Ball, R. Haemmerle, S. Naundorf, K. Kuhlcke, M. Rose, C. Fraser, L. Mathias, R. Ferrari, M.R. Abboud, W. Al-Herz, I. Kondratenko, L. Marodi, H. Glimm, B. Schlegelberger, A. Schambach, M.H. Albert, M. Schmidt, C. von Kalle, C. Klein, Gene therapy for Wiskott-Aldrich syndrome—long-term efficacy and genotoxicity, Sci. Transl. Med. 6 (2014) 227ra233.
[64] H.J. Kang, C.C. Bartholomae, A. Paruzynski, A. Arens, S. Kim, S.S. Yu, Y. Hong, C.W. Joo, N.K. Yoon, J.W. Rhim, J.G. Kim, C. Von Kalle, M. Schmidt, S. Kim, H.S. Ahn, Retroviral gene therapy for X-linked chronic granulomatous disease: results from phase I/II trial, Mol. Ther. 19 (2011) 2092–2101.
[65] D.B. Kohn, Historical perspective on the current renaissance for hematopoietic stem cell gene therapy, Hematol. Oncol. Clin. North Am. 31 (2017) 721–735.
[66] G. Ferrari, M. Cavazzana, F. Mavilio, Gene therapy approaches to hemoglobinopathies, Hematol. Oncol. Clin. North Am. 31 (2017) 835–852.
[67] J.L. Kwiatkowski, A.A. Thompson, J. Rasko, S. Hongeng, G.J. Schiller, U. Anurathapan, M. Cavazzana, P.J. Ho, C. von Kalle, M. Kletzel, P. Leboulch, E. Vichinsky, B. Deary, M. Asmal, M.C. Walters, Clinical outcomes up to 3 years following lentiglobin gene therapy for transfusion-dependent β-thalassemia in the Northstar Hgb-204 study, 59th ASH Annual Meeting & Exposition, 2017.
[68] A.A. Thompson, J.L. Kwiatkowski, J. Rasko, S. Hongeng, G.J. Schiller, U. Anurathapan, M. Cavazzana, P.J. Ho, C. von Kalle, M. Kletzel, P. Leboulch, E. Vichinsky, A. Petrusich, M. Asmal, M.C. Walters, Lentiglobin gene therapy for transfusion-dependent β-thalassemia: update from the Northstar Hgb-204 phase 1/2 clinical study, 58th ASH Annual Meeting & Exposition, Blood, 2016, p. 1175.
[69] J.A. Ribeil, S. Hacein-Bey-Abina, E. Payen, M. Semeraro, M. Elisa, L. Caccavelli, F. Touzot, F. Lefrere, F. Suarez, O. Hermine, V. Brousse, C. Poirot, B. Neven, P. Bourget, W. El Nemer, P. Bartolucci, A.L. Weber-Benarous, H. Puy, J.-F. Méritet, D. Grevent, Y. Beuzard, S. Chrétien, T. Lefebvre, M. Asmal, M. Joseney-Antoine, M. de Montalembert, S. Blanche, P. Leboulch, M. Cavazzana, Update from the Hgb-205 phase 1/2 clinical study of lentiglobin gene therapy: sustained clinical benefit in severe hemoglobinopathies, 58th ASH Annual Meeting & Exposition, Blood, 2016, p. 2311.
[70] J.A. Ribeil, S. Hacein-Bey-Abina, E. Payen, M. Elisa, A. Magnani, M. Semeraro, L. Caccavelli, F. Touzot, F. Lefrere, F. Suarez, O. Hermine, V. Brousse, C. Poirot, D. Moshous, P. Bourget, W. El-Nemer, P. Bartolucci, L. Weber, H. Puy, J.-F. Méritet, D. Grevent, Y. Beuzard, S. Chrétien, T. Lefebvre, M. Asmal, L. Sandler, M. de Montalembert, S. Blanche, P. Leboulch, M. Cavazzana, Update on the first patients with severe hemoglobinopathies treated with lentiglobin gene therapy, 22th EHA, 2017.
[71] J. Mansilla-Soto, I. Riviere, F. Boulad, M. Sadelain, Cell and gene therapy for the beta-thalassemias: advances and prospects, Hum. Gene Ther. 27 (2016) 295–304.
[72] M.E. Bernardo, E. Piras, A. Vacca, G. Giorgiani, M. Zecca, A. Bertaina, D. Pagliara, B. Contoli, R.M. Pinto, G. Caocci, A. Mastronuzzi, G. La Nasa, F. Locatelli, Allogeneic hematopoietic stem cell transplantation in thalassemia major: results of a reduced-toxicity conditioning regimen based on the use of treosulfan, Blood 120 (2012) 473–476.
[73] S. Scaramuzza, S. Marktel, M.P. Cicalese, F. Giglio, V. Calbi, M. Casiraghi, M.R. Lidonnici, C. Rossi, G. Mandelli, R. Milani, S. Gattillo, A. Calabria, E. Montini, G. Graziadei, L. Naldini, M.D. Cappellini, A. Aiuti, F. Ciceri, G. Ferrari, Gene therapy for beta thalassemia: initial results from TIGET BTHAL clinical trial, ASGCT's 20th Annual Meeting, Molecular Therapy, 2017, p. 331.
[74] B.K. Adler, D.E. Salzman, M.H. Carabasi, W.P. Vaughan, V.V. Reddy, J.T. Prchal, Fatal sickle cell crisis after granulocyte colony-stimulating factor administration, Blood 97 (2001) 3313–3314.
[75] J.A. Ribeil, S. Hacein-Bey-Abina, E. Payen, A. Magnani, M. Semeraro, E. Magrin, L. Caccavelli, B. Neven, P. Bourget, W. El Nemer, P. Bartolucci, L. Weber, H. Puy, J.F. Meritet, D. Grevent, Y. Beuzard, S. Chretien, T. Lefebvre, R.W. Ross, O. Negre, G. Veres, L. Sandler, S. Soni, M. de Montalembert, S. Blanche, P. Leboulch, M. Cavazzana, Gene therapy in a patient with sickle cell disease, N. Engl. J. Med. 376 (2017) 848–855.
[76] Bluebird Bio to Present New Data from Clinical Studies of LentiGlobinTM Gene Therapy in Transfusion-Dependent β-Thalassemia and Severe Sickle Cell Disease and bb2121 in Relapsed/Refractory Multiple Myeloma at ASH Annual Meeting, from, 2017. http://www.businesswire.com/news/home/20171101005861/en/, .
[77] J. Kanter, M.C. Walters, M. Hsieh, A.A. Thompson, L. Krishnamurti, J. Kwiatkowski, R.T. Kamble, C. von Kalle, F.A. Kuypers, M. Cavazzana, P. Leboulch, L. Sandler, S. Soni, J.F. Tisdale, Interim results from a phase 1/2 clinical study of lentiglobin gene therapy for severe sickle cell disease, 58th ASH Annual Meeting & Exposition, Blood, 2016, p. 1176.
[78] J. Kanter, M.C. Walters, M. Hsieh, L. Krishnamurti, J. Kwiatkowski, R.T. Kamble, C. von Kalle, M. Joseney-Antoine, F.J. Pierciey, P. Shi, M. Asmal, A.A. Thompson, J.F. Tisdale, Interim results from a phase 1/2 clinical study of lentiglobin gene therapy for severe sickle cell disease, 59th ASH Annual Meeting & Exposition, 2017.
[79] J.F. Tisdale, F.J. Pierciey, R. Kamble, J. Kanter, L. Krishnamurti, J.L. Kwiatkowski, A.A. Thompson, I. Shestopalov, M. Bonner, M. Joseney-Antoine, M. Asmal, M.C. Walters, Successful plerixafor-mediated mobilization, apheresis, and lentiviral vector transduction of hematopoietic stem cells in patients with severe sickle cell disease, 59th ASH Annual Meeting & Exposition, 2017.
[80] E. De Clercq, The bicyclam AMD3100 story, Nat. Rev. Drug Discov. 2 (2003) 581–587.
[81] J.F. DiPersio, E.A. Stadtmauer, A. Nademanee, I.N. Micallef, P.J. Stiff, J.L. Kaufman, R.T. Maziarz, C. Hosing, S. Fruehauf, M. Horwitz, D. Cooper, G. Bridger, G. Calandra, Plerixafor and G-CSF versus placebo and G-CSF to mobilize hematopoietic stem cells for autologous stem cell transplantation in patients with multiple myeloma, Blood 113 (2009) 5720–5726.
[82] E. Yannaki, T. Papayannopoulou, E. Jonlin, F. Zervou, G. Karponi, A. Xagorari, P. Becker, N. Psatha, I. Batsis, P. Kaloyannidis, V. Tahynopoulou, V. Constantinou, A. Bouinta, K. Kotta, A. Athanassiadou, A. Anagnostopoulos, A. Fassas, G. Stamatoyannopoulos, Hematopoietic stem cell mobilization for gene therapy of adult patients with severe beta-thalassemia: results of clinical trials using G-CSF or plerixafor in splenectomized and nonsplenectomized subjects, Mol. Ther. 20 (2012) 230–238.
[83] E. Yannaki, G. Karponi, F. Zervou, V. Constantinou, A. Bouinta, V. Tachynopoulou, K. Kotta, E. Jonlin, T. Papayannopoulou, A. Anagnostopoulos, G. Stamatoyannopoulos, Hematopoietic stem cell mobilization for gene therapy: superior mobilization by the combination of granulocyte-colony stimulating factor plus plerixafor in patients with beta-thalassemia major, Hum. Gene Ther. 24 (2013) 852–860.
[84] G. Karponi, N. Psatha, C.W. Lederer, J.E. Adair, F. Zervou, N. Zogas, M. Kleanthous, C. Tsatalas, A. Anagnostopoulos, M. Sadelain, I. Riviere, G. Stamatoyannopoulos, E. Yannaki, Plerixafor + G-CSF-mobilized CD34 + cells represent an optimal graft source for thalassemia gene therapy, Blood 126 (2015) 616–619.
[85] M.R. Lidonnici, S. Scaramuzza, A. Aprile, C. Rossi, G. Mandelli, S. Marktel, F. Ciceri, B. Gentner, A. Calabria, E. Montini, G. Ferrari, Addition of dmPGE2 enhances BM and mPB CD34 + cell transduction preserving in vivo reconstitution potential, differentiation and integration profile of Globin-LV, ASGCT's 20th Annual Meeting, Molecular Therapy, 2017, p. 90.
[86] C.D. Fitzhugh, M.M. Hsieh, C.D. Bolan, C. Saenz, J.F. Tisdale, Granulocyte colony-stimulating factor (G-CSF) administration in individuals with sickle cell disease: time for a moratorium? Cytotherapy 11 (2009) 464–471.
[87] F. Boulad, T.B. Shore, K. Van Besien, D. Guarneri, J. Greenberg, C. Minniti, S. Wiener Fedus, F. Perna, X. Wang, I. Riviere, M. Sadelain, P. Shi, Safety and efficacy trial of escalation of plerixafor for mobilization of CD34 + Hematopoietic Progenitor Cells (HPCs) for globin gene transfer in patients with sickle cell disease, 59th ASH Annual Meeting & Exposition, 2017.
[88] A. Aiuti, L. Biasco, S. Scaramuzza, F. Ferrua, M.P. Cicalese, C. Baricordi, F. Dionisio, A. Calabria, S. Giannelli, M.C. Castiello, M. Bosticardo, C. Evangelio, A. Assanelli, M. Casiraghi, S. Di Nunzio, L. Callegaro, C. Benati, P. Rizzardi, D. Pellin, C. Di Serio, M. Schmidt, C. Von Kalle, J. Gardner, N. Mehta, V. Neduva, D.J. Dow, A. Galy, R. Miniero, A. Finocchi, A. Metin, P.P. Banerjee, J.S. Orange, S. Galimberti, M.G. Valsecchi, A. Biffi, E. Montini, A. Villa, F. Ciceri, M.G. Roncarolo, L. Naldini, Lentiviral hematopoietic stem cell gene therapy in

patients with Wiskott-Aldrich syndrome, Science 341 (2013) 1233151.
[89] A. Biffi, E. Montini, L. Lorioli, M. Cesani, F. Fumagalli, T. Plati, C. Baldoli, S. Martino, A. Calabria, S. Canale, F. Benedicenti, G. Vallanti, L. Biasco, S. Leo, N. Kabbara, G. Zanetti, W.B. Rizzo, N.A. Mehta, M.P. Cicalese, M. Casiraghi, J.J. Boelens, U. Del Carro, D.J. Dow, M. Schmidt, A. Assanelli, V. Neduva, C. Di Serio, E. Stupka, J. Gardner, C. von Kalle, C. Bordignon, F. Ciceri, A. Rovelli, M.G. Roncarolo, A. Aiuti, M. Sessa, L. Naldini, Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy, Science 341 (2013) 1233158.
[90] L. Biasco, D. Pellin, S. Scala, F. Dionisio, L. Basso-Ricci, L. Leonardelli, S. Scaramuzza, C. Baricordi, F. Ferrua, M.P. Cicalese, S. Giannelli, V. Neduva, D.J. Dow, M. Schmidt, C. Von Kalle, M.G. Roncarolo, F. Ciceri, P. Vicard, E. Wit, C. Di Serio, L. Naldini, A. Aiuti, In vivo tracking of human hematopoiesis reveals patterns of clonal dynamics during early and steady-state reconstitution phases, Cell Stem Cell 19 (2016) 107–119.
[91] K. Baldwin, F. Urbinati, Z. Romero, B. Campo-Fernandez, M.L. Kaufman, A.R. Cooper, K. Masiuk, R.P. Hollis, D.B. Kohn, Enrichment of human hematopoietic stem/progenitor cells facilitates transduction for stem cell gene therapy, Stem Cells 33 (2015) 1532–1542.
[92] G. Tricot, Y. Gazitt, T. Leemhuis, S. Jagannath, K.R. Desikan, D. Siegel, A. Fassas, S. Tindle, J. Nelson, C. Juttner, A. Tsukamoto, J. Hallagan, K. Atkinson, C. Reading, R. Hoffman, B. Barlogie, Collection, tumor contamination, and engraftment kinetics of highly purified hematopoietic progenitor cells to support high dose therapy in multiple myeloma, Blood 91 (1998) 4489–4495.
[93] R.S. Negrin, K. Atkinson, T. Leemhuis, E. Hanania, C. Juttner, K. Tierney, W.W. Hu, L.J. Johnston, J.A. Shizurn, K.E. Stockerl-Goldstein, K.G. Blume, I.L. Weissman, S. Bower, R. Baynes, R. Dansey, C. Karanes, W. Peters, J. Klein, Transplantation of highly purified CD34 + Thy-1 + hematopoietic stem cells in patients with metastatic breast cancer, Biol. Blood Marrow Transplant. 6 (2000) 262–271.
[94] M. Michallet, T. Philip, I. Philip, H. Godinot, C. Sebban, G. Salles, A. Thiebaut, P. Biron, F. Lopez, P. Mazars, N. Roubi, T. Leemhuis, E. Hanania, C. Reading, G. Fine, K. Atkinson, C. Juttner, B. Coiffier, D. Fiere, E. Archimbaud, Transplantation with selected autologous peripheral blood CD34 + Thy1 + hematopoietic stem cells (HSCs) in multiple myeloma: impact of HSC dose on engraftment, safety, and immune reconstitution, Exp. Hematol. 28 (2000) 858–870.
[95] E. Zonari, G. Desantis, C. Petrillo, F.E. Boccalatte, M.R. Lidonnici, A. Kajaste-Rudnitski, A. Aiuti, G. Ferrari, L. Naldini, B. Gentner, Efficient ex vivo engineering and expansion of highly purified human hematopoietic stem and progenitor cell populations for gene therapy, Stem Cell Rep. 8 (2017) 977–990.
[96] K.E. Masiuk, D. Brown, J. Laborada, R.P. Hollis, F. Urbinati, D.B. Kohn, Improving gene therapy efficiency through the enrichment of human hematopoietic stem cells, Mol. Ther. 25 (2017) 2163–2175.
[97] S.P. McDermott, K. Eppert, E.R. Lechman, M. Doedens, J.E. Dick, Comparison of human cord blood engraftment between immunocompromised mouse strains, Blood 116 (2010) 193–200.
[98] F. Notta, S. Doulatov, J.E. Dick, Engraftment of human hematopoietic stem cells is more efficient in female NOD/SCID/IL-2Rgc-null recipients, Blood 115 (2010) 3704–3707.
[99] C.C. Zhang, M. Kaba, G. Ge, K. Xie, W. Tong, C. Hug, H.F. Lodish, Angiopoietin-like proteins stimulate ex vivo expansion of hematopoietic stem cells, Nat. Med. 12 (2006) 240–245.
[100] C.C. Zhang, M. Kaba, S. Iizuka, H. Huynh, H.F. Lodish, Angiopoietin-like 5 and IGFBP2 stimulate ex vivo expansion of human cord blood hematopoietic stem cells as assayed by NOD/SCID transplantation, Blood 111 (2008) 3415–3423.
[101] A.E. Boitano, J. Wang, R. Romeo, L.C. Bouchez, A.E. Parker, S.E. Sutton, J.R. Walker, C.A. Flaveny, G.H. Perdew, M.S. Denison, P.G. Schultz, M.P. Cooke, Aryl hydrocarbon receptor antagonists promote the expansion of human hematopoietic stem cells, Science 329 (2010) 1345–1348.
[102] C. Delaney, S. Heimfeld, C. Brashem-Stein, H. Voorhies, R.L. Manger, I.D. Bernstein, Notch-mediated expansion of human cord blood progenitor cells capable of rapid myeloid reconstitution, Nat. Med. 16 (2010) 232–236.
[103] I. Fares, J. Chagraoui, Y. Gareau, S. Gingras, R. Ruel, N. Mayotte, E. Csaszar, D.J. Knapp, P. Miller, M. Ngom, S. Imren, D.C. Roy, K.L. Watts, H.P. Kiem, R. Herrington, N.N. Iscove, R.K. Humphries, C.J. Eaves, S. Cohen, A. Marinier, P.W. Zandstra, G. Sauvageau, Cord blood expansion. Pyrimidoindole derivatives are agonists of human hematopoietic stem cell self-renewal, Science 345 (2014) 1509–1512.
[104] T.E. North, W. Goessling, C.R. Walkley, C. Lengerke, K.R. Kopani, A.M. Lord, G.J. Weber, T.V. Bowman, I.H. Jang, T. Grosser, G.A. Fitzgerald, G.Q. Daley, S.H. Orkin, L.I. Zon, Prostaglandin E2 regulates vertebrate haematopoietic stem cell homeostasis, Nature 447 (2007) 1007–1011.
[105] W. Goessling, R.S. Allen, X. Guan, P. Jin, N. Uchida, M. Dovey, J.M. Harris, M.E. Metzger, A.C. Bonifacino, D. Stroncek, J. Stegner, M. Armant, T. Schlaeger, J.F. Tisdale, L.I. Zon, R.E. Donahue, T.E. North, Prostaglandin E2 enhances human cord blood stem cell xenotransplants and shows long-term safety in preclinical nonhuman primate transplant models, Cell Stem Cell 8 (2011) 445–458.
[106] G.C. Heffner, M. Bonner, L. Christiansen, F.J. Pierciey, D. Campbell, Y. Smurnyy, W. Zhang, A. Hamel, S. Shaw, G. Lewis, K.A. Goss, O. Garijo, B.E. Torbett, H. Horton, M.H. Finer, P.D. Gregory, G. Veres, Prostaglandin E2 increases lentiviral vector transduction efficiency of adult human hematopoietic stem and progenitor cells, Mol. Ther. 26 (2018) 320–328.
[107] C.X. Wang, B.D. Sather, X. Wang, J. Adair, I. Khan, S. Singh, S. Lang, A. Adams, G. Curinga, H.P. Kiem, C.H. Miao, D.J. Rawlings, B.E. Torbett, Rapamycin relieves lentiviral vector transduction resistance in human and mouse hematopoietic stem cells, Blood 124 (2014) 913–923.
[108] C. Petrillo, D. Cesana, F. Piras, S. Bartolaccini, L. Naldini, E. Montini, A. Kajaste-Rudnitski, Cyclosporin a and rapamycin relieve distinct lentiviral restriction blocks in hematopoietic stem and progenitor cells, Mol. Ther. 23 (2015) 352–362.
[109] F. Frassoni, F. Gualandi, M. Podesta, A.M. Raiola, A. Ibatici, G. Piaggio, M. Sessarego, N. Sessarego, M. Gobbi, N. Sacchi, M. Labopin, A. Bacigalupo, Direct intrabone transplant of unrelated cord-blood cells in acute leukaemia: a phase I/II study, Lancet Oncol. 9 (2008) 831–839.
[110] M. Cavazzana-Calvo, S. Hacein-Bey, G. de Saint Basile, F. Gross, E. Yvon, P. Nusbaum, F. Selz, C. Hue, S. Certain, J.L. Casanova, P. Bousso, F.L. Deist, A. Fischer, Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease, Science 288 (2000) 669–672.
[111] S. Hacein-Bey Abina, H.B. Gaspar, J. Blondeau, L. Caccavelli, S. Charrier, K. Buckland, C. Picard, E. Six, N. Himoudi, K. Gilmour, A.M. McNicol, H. Hara, J. Xu-Bayford, C. Rivat, F. Touzot, F. Mavilio, A. Lim, J.M. Treluyer, S. Heritier, F. Lefrere, J. Magalon, I. Pengue-Koyi, G. Honnet, S. Blanche, E.A. Sherman, F. Male, C. Berry, N. Malani, F.D. Bushman, A. Fischer, A.J. Thrasher, A. Galy, M. Cavazzana, Outcomes following gene therapy in patients with severe Wiskott-Aldrich syndrome, JAMA 313 (2015) 1550–1563.
[112] E.C. Morris, T. Fox, R. Chakraverty, R. Tendeiro, K. Snell, C. Rivat, S. Grace, K. Gilmour, S. Workman, K. Buckland, K. Butler, R. Chee, A.D. Salama, H. Ibrahim, H. Hara, C. Duret, F. Mavilio, F. Male, F.D. Bushman, A. Galy, S.O. Burns, H.B. Gaspar, A.J. Thrasher, Gene therapy for Wiskott-Aldrich syndrome in a severely affected adult, Blood 130 (2017) 1327–1335.
[113] M. Sessa, L. Lorioli, F. Fumagalli, S. Acquati, D. Redaelli, C. Baldoli, S. Canale, I.D. Lopez, F. Morena, A. Calabria, R. Fiori, P. Silvani, P.M. Rancoita, M. Gabaldo, F. Benedicenti, G. Antonioli, A. Assanelli, M.P. Cicalese, U. Del Carro, M.G. Sora, S. Martino, A. Quattrini, E. Montini, C. Di Serio, F. Ciceri, M.G. Roncarolo, A. Aiuti, L. Naldini, A. Biffi, Lentiviral haemopoietic stem-cell gene therapy in early-onset metachromatic leukodystrophy: an ad-hoc analysis of a non-randomised, open-label, phase 1/2 trial, Lancet 388 (2016) 476–487.
[114] D. Kohn, A Phase I/II clinical trial opened in September 2015 for X-linked chronic granulomatous diseases, Changing the Face of Modern Medicine: Stem Cells and Gene Therapy, Human Gene Therapy, Florence, Italy, 2016, p. A17.
[115] F. Centis, L. Tabellini, G. Lucarelli, O. Buffi, P. Tonucci, B. Persini, M. Annibali, R. Emiliani, A. Iliescu, S. Rapa, R. Rossi, L. Ma, E. Angelucci, S.L. Schrier, The importance of erythroid expansion in determining the extent of apoptosis in erythroid precursors in patients with beta-thalassemia major, Blood 96 (2000) 3624–3629.
[116] M.E. Bernardo, A. Aiuti, The role of conditioning in hematopoietic stem cell gene therapy, Hum. Gene Ther. 27 (2016) 741–748.
[117] A. Aiuti, L. Naldini, Safer conditioning for blood stem cell transplants, Nat. Biotechnol. 34 (2016) 721–723.
[118] M.J. Cowan, C.C. Dvorak, J. Long-Boyle, Opening marrow niches in patients undergoing autologous hematopoietic stem cell gene therapy, Hematol. Oncol. Clin. North Am. 31 (2017) 809–822.
[119] A. Czechowicz, D. Kraft, I.L. Weissman, D. Bhattacharya, Efficient transplantation via antibody-based clearance of hematopoietic stem cell niches, Science 318 (2007) 1296–1299.
[120] X. Xue, N.K. Pech, W.C. Shelley, E.F. Srour, M.C. Yoder, M.C. Dinauer, Antibody targeting KIT as pretransplantation conditioning in immunocompetent mice, Blood 116 (2010) 5419–5422.
[121] K.C. Straathof, K. Rao, M. Eyrich, G. Hale, P. Bird, E. Berrie, L. Brown, S. Adams, P.G. Schlegel, N. Goulden, H.B. Gaspar, A.R. Gennery, P. Landais, E.G. Davies, M.K. Brenner, P.A. Veys, P.J. Amrolia, Haemopoietic stem-cell transplantation with antibody-based minimal-intensity conditioning: a phase 1/2 study, Lancet 374 (2009) 912–920.
[122] R. Palchaudhuri, B. Saez, J. Hoggatt, A. Schajnovitz, D.B. Sykes, T.A. Tate, A. Czechowicz, Y. Kfoury, F. Ruchika, D.J. Rossi, G.L. Verdine, M.K. Mansour, D.T. Scadden, Non-genotoxic conditioning for hematopoietic stem cell transplantation using a hematopoietic-cell-specific internalizing immunotoxin, Nat. Biotechnol. 34 (2016) 738–745.
[123] A. Chhabra, A.M. Ring, K. Weiskopf, P.J. Schnorr, S. Gordon, A.C. Le, H.S. Kwon, N.G. Ring, J. Volkmer, P.Y. Ho, S. Tseng, I.L. Weissman, J.A. Shizuru, Hematopoietic stem cell transplantation in immunocompetent hosts without radiation or chemotherapy, Sci. Transl. Med. 8 (2016) 351ra105.
[124] S. Chandrakasan, R. Jayavaradhan, J. Ernst, A. Shrestha, A. Loberg, P. Dexheimer, M. Jordan, Q. Pang, B. Aronow, P. Malik, KIT blockade is sufficient for donor hematopoietic stem cell engraftment in Fanconi anemia mice, Blood 129 (2017) 1048–1052.
[125] A.S. Artz, From biology to clinical practice: aging and hematopoietic cell transplantation, Biol. Blood Marrow Transplant. 18 (2012) S40–45.
[126] A.R. Rezvani, B.E. Storer, K.A. Guthrie, H.G. Schoch, D.G. Maloney, B.M. Sandmaier, R. Storb, Impact of donor age on outcome after allogeneic hematopoietic cell transplantation, Biol. Blood Marrow Transplant. 21 (2015) 105–112.
[127] C. Kollman, C.W. Howe, C. Anasetti, J.H. Antin, S.M. Davies, A.H. Filipovich, J. Hegland, N. Kamani, N.A. Kernan, R. King, V. Ratanatharathorn, D. Weisdorf, D.L. Confer, Donor characteristics as risk factors in recipients after transplantation of bone marrow from unrelated donors: the effect of donor age, Blood 98 (2001) 2043–2051.
[128] S.J. Morrison, A.M. Wandycz, K. Akashi, A. Globerson, I.L. Weissman, The aging of hematopoietic stem cells, Nat. Med. 2 (1996) 1011–1016.
[129] G. de Haan, G. Van Zant, Dynamic changes in mouse hematopoietic stem cell numbers during aging, Blood 93 (1999) 3294–3301.
[130] D.E. Harrison, C.M. Astle, Loss of stem cell repopulating ability upon transplantation. Effects of donor age, cell number, and transplantation procedure, J. Exp.

Med. 156 (1982) 1767–1779.
[131] H. Geiger, G. Van Zant, The aging of lympho-hematopoietic stem cells, Nat. Immunol. 3 (2002) 329–333.
[132] S.E. Latchney, L.M. Calvi, The aging hematopoietic stem cell niche: phenotypic and functional changes and mechanisms that contribute to hematopoietic aging, Semin. Hematol. 54 (2017) 25–32.
[133] P. Mombaerts, J. Iacomini, R.S. Johnson, K. Herrup, S. Tonegawa, V.E. Papaioannou, RAG-1-deficient mice have no mature B and T lymphocytes, Cell 68 (1992) 869–877.
[134] Y. Shinkai, G. Rathbun, K.P. Lam, E.M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A.M. Stall, et al., RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement, Cell 68 (1992) 855–867.
[135] D. Moshous, I. Callebaut, R. de Chasseval, B. Corneo, M. Cavazzana-Calvo, F. Le Deist, I. Tezcan, O. Sanal, Y. Bertrand, N. Philippe, A. Fischer, J.P. de Villartay, Artemis, a novel DNA double-strand break repair/V(D)J recombination protein, is mutated in human severe combined immune deficiency, Cell 105 (2001) 177–186.
[136] A.V. Sauer, A. Aiuti, New insights into the pathogenesis of adenosine deaminase-severe combined immunodeficiency and progress in gene therapy, Curr. Opin. Allergy Clin. Immunol. 9 (2009) 496–502.
[137] R. Sokolic, I. Maric, C. Kesserwan, E. Garabedian, I.C. Hanson, M. Dodds, R. Buckley, A.C. Issekutz, N. Kamani, K. Shaw, B. Tan, P. Bali, M.S. Hershfield, D.B. Kohn, A.S. Wayne, F. Candotti, Myeloid dysplasia and bone marrow hypocellularity in adenosine deaminase-deficient severe combined immune deficiency, Blood 118 (2011) 2688–2694.
[138] M.P. Cicalese, F. Ferrua, L. Castagnaro, R. Pajno, F. Barzaghi, S. Giannelli, F. Dionisio, I. Brigida, M. Bonopane, M. Casiraghi, A. Tabucchi, F. Carlucci, E. Grunebaum, M. Adeli, R.G. Bredius, J.M. Puck, P. Stepensky, I. Tezcan, K. Rolfe, E. De Boever, R.R. Reinhardt, J. Appleby, F. Ciceri, M.G. Roncarolo, A. Aiuti, Update on the safety and efficacy of retroviral gene therapy for immunodeficiency due to adenosine deaminase deficiency, Blood 128 (2016) 45–54.
[139] C. Lacout, E. Haddad, S. Sabri, F. Svinarchouk, L. Garcon, C. Capron, A. Foudi, R. Mzali, S.B. Snapper, F. Louache, W. Vainchenker, D. Dumenil, A defect in hematopoietic stem cell migration explains the nonrandom X-chromosome inactivation in carriers of Wiskott-Aldrich syndrome, Blood 102 (2003) 1282–1289.
[140] N. Starc, D. Ingo, A. Conforti, V. Rossella, L. Tomao, A. Pitisci, F. De Mattia, I. Brigida, M. Algeri, M. Montanari, G. Palumbo, P. Merli, P. Rossi, A. Aiuti, F. Locatelli, M.E. Bernardo, Biological and functional characterization of bone marrow-derived mesenchymal stromal cells from patients affected by primary immunodeficiency, Sci. Rep. 7 (2017) 8153.
[141] A.L. Aprile, M.R. Lidonnici, G. Mandelli, A. Gulino, C. Tripodo, G. Ferrari, Thalassemic bone marrow microenvironment accelerates hematopoietic stem cell ageing and exhaustion, Changing the Face of Modern Medicine: Stem Cells and Gene Therapy, Human Gene Therapy. Nov 2016, 2016, p. A38.
[142] J.A. Grasso, A.L. Sullivan, L.W. Sullivan, Ultrastructural studies of the bone marrow in sickle cell anaemia. II. The morphology of erythropoietic cells and their response to deoxygenation in vitro, Br. J. Haematol. 31 (1975) 381–389.
[143] J.A. Grasso, A.L. Sullivan, L.W. Sullivan, Ultrastructural studies of the bone marrow in sickle cell anaemia. I. The structure of sickled erythrocytes and reticulocytes and their phagocytic destruction, Br. J. Haematol. 31 (1975) 135–148.
[144] G.J. Kato, New insights into sickle cell disease: mechanisms and investigational therapies, Curr. Opin. Hematol. 23 (2016) 224–232.
[145] Y. Shiozawa, Y. Jung, A.M. Ziegler, E.A. Pedersen, J. Wang, Z. Wang, J. Song, J. Wang, C.H. Lee, S. Sud, K.J. Pienta, P.H. Krebsbach, R.S. Taichman, Erythropoietin couples hematopoiesis with bone formation, PLoS One 5 (2010) e10853.
[146] J.F. Whitfield, Parathyroid hormone and leptin—new peptides, expanding clinical prospects, Expert Opin. Investig. Drugs 14 (2005) 251–264.
[147] N.V. Petrova, D.A. Svinareva, I.N. Nifontova, K.S. Momotyuk, V.G. Savchenko, N.I. Drize, Stromal regulation of hemopoietic stem cells in long-term human bone marrow tissue cultures under the effect of parathyroid hormone, Bull. Exp. Biol. Med. 142 (2006) 527–530.
[148] L.M. Calvi, G.B. Adams, K.W. Weibrecht, J.M. Weber, D.P. Olson, M.C. Knight, R.P. Martin, E. Schipani, P. Divieti, F.R. Bringhurst, L.A. Milner, H.M. Kronenberg, D.T. Scadden, Osteoblastic cells regulate the haematopoietic stem cell niche, Nature 425 (2003) 841–846.
[149] R. Crichton, Iron Metabolism: From Molecular Mechanisms to Clinical Consequences, 4th Edition, John Wiley and Sons, 2009.
[150] A. Ludin, S. Gur-Cohen, K. Golan, K.B. Kaufmann, T. Itkin, C. Medaglia, X.J. Lu, G. Ledergor, O. Kollet, T. Lapidot, Reactive oxygen species regulate hematopoietic stem cell self-renewal, migration and development, as well as their bone marrow microenvironment, Antioxid. Redox Signal. 21 (2014) 1605–1619.
[151] A. Wilson, E. Laurenti, G. Oser, R.C. van der Wath, W. Blanco-Bose, M. Jaworski, S. Offner, C.F. Dunant, L. Eshkind, E. Bockamp, P. Lio, H.R. Macdonald, A. Trumpp, Hematopoietic stem cells reversibly switch from dormancy to self-renewal during homeostasis and repair, Cell 135 (2008) 1118–1129.
[152] J. Westra, E. Brouwer, R. Bos, M.D. Posthumus, B. Doornbos-van der Meer, C.G. Kallenberg, P.C. Limburg, Regulation of cytokine-induced HIF-1alpha expression in rheumatoid synovial fibroblasts, Ann. N. Y. Acad. Sci. 1108 (2007) 340–348.
[153] M.M. Juntilla, V.D. Patil, M. Calamito, R.P. Joshi, M.J. Birnbaum, G.A. Koretzky, AKT1 and AKT2 maintain hematopoietic stem cell function by regulating reactive oxygen species, Blood 115 (2010) 4030–4038.
[154] K. Ito, A. Hirao, F. Arai, K. Takubo, S. Matsuoka, K. Miyamoto, M. Ohmura, K. Naka, K. Hosokawa, Y. Ikeda, T. Suda, Reactive oxygen species act through p38 MAPK to limit the lifespan of hematopoietic stem cells, Nat. Med. 12 (2006) 446–451.
[155] Y.Y. Jang, S.J. Sharkis, A low level of reactive oxygen species selects for primitive hematopoietic stem cells that may reside in the low-oxygenic niche, Blood 110 (2007) 3056–3063.
[156] K. Ito, A. Hirao, F. Arai, S. Matsuoka, K. Takubo, I. Hamaguchi, K. Nomiyama, K. Hosokawa, K. Sakurada, N. Nakagata, Y. Ikeda, T.W. Mak, T. Suda, Regulation of oxidative stress by ATM is required for self-renewal of haematopoietic stem cells, Nature 431 (2004) 997–1002.
[157] H. Okabe, T. Suzuki, E. Uehara, M. Ueda, T. Nagai, K. Ozawa, The bone marrow hematopoietic microenvironment is impaired in iron-overloaded mice, Eur. J. Haematol. 93 (2014) 118–128.
[158] T.I. Cornu, C. Mussolino, T. Cathomen, Refining strategies to translate genome editing to the clinic, Nat. Med. 23 (2017) 415–423.
[159] M. Jinek, K. Chylinski, I. Fonfara, M. Hauer, J.A. Doudna, E. Charpentier, A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity, Science 337 (2012) 816–821.
[160] F. Chen, S.M. Pruett-Miller, Y. Huang, M. Gjoka, K. Duda, J. Taunton, T.N. Collingwood, M. Frodin, G.D. Davis, High-frequency genome editing using ssDNA oligonucleotides with zinc-finger nucleases, Nat. Methods 8 (2011) 753–755.
[161] M.L. Hirsch, L. Green, M.H. Porteus, R.J. Samulski, Self-complementary AAV mediates gene targeting and enhances endonuclease delivery for double-strand break repair, Gene Ther. 17 (2010) 1175–1180.
[162] W. Deng, J.W. Rupon, I. Krivega, L. Breda, I. Motta, K.S. Jahn, A. Reik, P.D. Gregory, S. Rivella, A. Dean, G.A. Blobel, Reactivation of developmentally silenced globin genes by forced chromatin looping, Cell 158 (2014) 849–860.
[163] N. Sun, H. Zhao, Seamless correction of the sickle cell disease mutation of the HBB gene in human induced pluripotent stem cells using TALENs, Biotechnol. Bioeng. 111 (2014) 1048–1053.
[164] M.D. Hoban, G.J. Cost, M.C. Mendel, Z. Romero, M.L. Kaufman, A.V. Joglekar, M. Ho, D. Lumaquin, D. Gray, G.R. Lill, A.R. Cooper, F. Urbinati, S. Senadheera, A. Zhu, P.Q. Liu, D.E. Paschon, L. Zhang, E.J. Rebar, A. Wilber, X. Wang, P.D. Gregory, M.C. Holmes, A. Reik, R.P. Hollis, D.B. Kohn, Correction of the sickle cell disease mutation in human hematopoietic stem/progenitor cells, Blood 125 (2015) 2597–2604.
[165] M.D. Hoban, D. Lumaquin, C.Y. Kuo, Z. Romero, J. Long, M. Ho, C.S. Young, M. Mojadidi, S. Fitz-Gibbon, A.R. Cooper, G.R. Lill, F. Urbinati, B. Campo-Fernandez, C.F. Bjurstrom, M. Pellegrini, R.P. Hollis, D.B. Kohn, CRISPR/Cas9-mediated correction of the sickle mutation in human CD34 + cells, Mol. Ther. 24 (2016) 1561–1569.
[166] S. Samakoglu, L. Lisowski, T. Budak-Alpdogan, Y. Usachenko, S. Acuto, R. Di Marzo, A. Maggio, P. Zhu, J.F. Tisdale, I. Riviere, M. Sadelain, A genetic strategy to treat sickle cell anemia by coregulating globin transgene expression and RNA interference, Nat. Biotechnol. 24 (2006) 89–94.
[167] C. Zuccato, L. Breda, F. Salvatori, G. Breveglieri, S. Gardenghi, N. Bianchi, E. Brognara, I. Lampronti, M. Borgatti, S. Rivella, R. Gambari, A combined approach for beta-thalassemia based on gene therapy-mediated adult hemoglobin (HbA) production and fetal hemoglobin (HbF) induction, Ann. Hematol. 91 (2012) 1201–1213.
[168] D.E. Bauer, S.C. Kamran, S.H. Orkin, Reawakening fetal hemoglobin: prospects for new therapies for the beta-globin disorders, Blood 120 (2012) 2945–2953.
[169] R.P. Patwardhan, J.B. Hiatt, D.M. Witten, M.J. Kim, R.P. Smith, D. May, C. Lee, J.M. Andrie, S.I. Lee, G.M. Cooper, N. Ahituv, L.A. Pennacchio, J. Shendure, Massively parallel functional dissection of mammalian enhancers in vivo, Nat. Biotechnol. 30 (2012) 265–270.
[170] A. Melnikov, A. Murugan, X. Zhang, T. Tesileanu, L. Wang, P. Rogov, S. Feizi, A. Gnirke, C.G. Callan Jr., J.B. Kinney, M. Kellis, E.S. Lander, T.S. Mikkelsen, Systematic dissection and optimization of inducible enhancers in human cells using a massively parallel reporter assay, Nat. Biotechnol. 30 (2012) 271–277.
[171] K.A. Frazer, S.S. Murray, N.J. Schork, E.J. Topol, Human genetic variation and its contribution to complex traits, Nat. Rev. Genet. 10 (2009) 241–251.
[172] D.E. Bauer, S.C. Kamran, S. Lessard, J. Xu, Y. Fujiwara, C. Lin, Z. Shao, M.C. Canver, E.C. Smith, L. Pinello, P.J. Sabo, J. Vierstra, R.A. Voit, G.C. Yuan, M.H. Porteus, J.A. Stamatoyannopoulos, G. Lettre, S.H. Orkin, An erythroid enhancer of BCL11A subject to genetic variation determines fetal hemoglobin level, Science 342 (2013) 253–257.
[173] M.C. Canver, E.C. Smith, F. Sher, L. Pinello, N.E. Sanjana, O. Shalem, D.D. Chen, P.G. Schupp, D.S. Vinjamur, S.P. Garcia, S. Luc, R. Kurita, Y. Nakamura, Y. Fujiwara, T. Maeda, G.C. Yuan, F. Zhang, S.H. Orkin, D.E. Bauer, BCL11A enhancer dissection by Cas9-mediated in situ saturating mutagenesis, Nature 527 (2015) 192–197.
[174] J. Vierstra, A. Reik, K.H. Chang, S. Stehling-Sun, Y. Zhou, S.J. Hinkley, D.E. Paschon, L. Zhang, N. Psatha, Y.R. Bendana, C.M. O'Neil, A.H. Song, A.K. Mich, P.Q. Liu, G. Lee, D.E. Bauer, M.C. Holmes, S.H. Orkin, T. Papayannopoulou, G. Stamatoyannopoulos, E.J. Rebar, P.D. Gregory, F.D. Urnov, J.A. Stamatoyannopoulos, Functional footprinting of regulatory DNA, Nat. Methods 12 (2015) 927–930.
[175] S. Luc, J. Huang, J.L. McEldoon, E. Somuncular, D. Li, C. Rhodes, S. Mamoor, S. Hou, J. Xu, S.H. Orkin, Bcl11a deficiency leads to hematopoietic stem cell defects with an aging-like phenotype, Cell Rep. 16 (2016) 3181–3194.
[176] C. Brendel, S. Guda, R. Renella, D.E. Bauer, M.C. Canver, Y.J. Kim, M.M. Heeney, D. Klatt, J. Fogel, M.D. Milsom, S.H. Orkin, R.I. Gregory, D.A. Williams, Lineage-specific BCL11A knockdown circumvents toxicities and reverses sickle phenotype, J. Clin. Invest. 126 (2016) 3868–3878.
[177] K.H. Chang, S.E. Smith, T. Sullivan, K. Chen, Q. Zhou, J.A. West, M. Liu, Y. Liu, B.F. Vieira, C. Sun, V.P. Hong, M. Zhang, X. Yang, A. Reik, F.D. Urnov, E.J. Rebar,

M.C. Holmes, O. Danos, H. Jiang, S. Tan, Long-term engraftment and fetal globin induction upon BCL11A gene editing in bone-marrow-derived CD34 + hematopoietic stem and progenitor cells, Mol. Ther. Methods Clin. Dev. 4 (2017) 137–148.

[178] M.A. DeWitt, W. Magis, N.L. Bray, T. Wang, J.R. Berman, F. Urbinati, S.J. Heo, T. Mitros, D.P. Munoz, D. Boffelli, D.B. Kohn, M.C. Walters, D. Carroll, D.I. Martin, J.E. Corn, Selection-free genome editing of the sickle mutation in human adult hematopoietic stem/progenitor cells, Sci. Transl. Med. 8 (2016) 360ra134.

[179] P. Genovese, G. Schiroli, G. Escobar, T. Di Tomaso, C. Firrito, A. Calabria, D. Moi, R. Mazzieri, C. Bonini, M.C. Holmes, P.D. Gregory, M. van der Burg, B. Gentner, E. Montini, A. Lombardo, L. Naldini, Targeted genome editing in human repopulating haematopoietic stem cells, Nature 510 (2014) 235–240.

[180] D.P. Dever, R.O. Bak, A. Reinisch, J. Camarena, G. Washington, C.E. Nicolas, M. Pavel-Dinu, N. Saxena, A.B. Wilkens, S. Mantri, N. Uchida, A. Hendel, A. Narla, R. Majeti, K.I. Weinberg, M.H. Porteus, CRISPR/Cas9 beta-globin gene targeting in human haematopoietic stem cells, Nature 539 (2016) 384–389.

[181] B. Wienert, A.P. Funnell, L.J. Norton, R.C. Pearson, L.E. Wilkinson-White, K. Lester, J. Vadolas, M.H. Porteus, J.M. Matthews, K.G. Quinlan, M. Crossley, Editing the genome to introduce a beneficial naturally occurring mutation associated with increased fetal globin, Nat. Commun. 6 (2015) 7085.

[182] E.A. Traxler, Y. Yao, Y.D. Wang, K.J. Woodard, R. Kurita, Y. Nakamura, J.R. Hughes, R.C. Hardison, G.A. Blobel, C. Li, M.J. Weiss, A genome-editing strategy to treat beta-hemoglobinopathies that recapitulates a mutation associated with a benign genetic condition, Nat. Med. 22 (2016) 987–990.

[183] Y. Fu, J.A. Foden, C. Khayter, M.L. Maeder, D. Reyon, J.K. Joung, J.D. Sander, High-frequency off-target mutagenesis induced by CRISPR-Cas nucleases in human cells, Nat. Biotechnol. 31 (2013) 822–826.

[184] R.L. Frock, J. Hu, R.M. Meyers, Y.J. Ho, E. Kii, F.W. Alt, Genome-wide detection of DNA double-stranded breaks induced by engineered nucleases, Nat. Biotechnol. 33 (2015) 179–186.

[185] R. Gabriel, A. Lombardo, A. Arens, J.C. Miller, P. Genovese, C. Kaeppel, A. Nowrouzi, C.C. Bartholomae, J. Wang, G. Friedman, M.C. Holmes, P.D. Gregory, H. Glimm, M. Schmidt, L. Naldini, C. von Kalle, An unbiased genome-wide analysis of zinc-finger nuclease specificity, Nat. Biotechnol. 29 (2011) 816–823.

[186] S.Q. Tsai, Z. Zheng, N.T. Nguyen, M. Liebers, V.V. Topkar, V. Thapar, N. Wyvekens, C. Khayter, A.J. Iafrate, L.P. Le, M.J. Aryee, J.K. Joung, GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases, Nat. Biotechnol. 33 (2015) 187–197.

[187] V. Pattanayak, J.P. Guilinger, D.R. Liu, Determining the specificities of TALENs, Cas9, and other genome-editing enzymes, Methods Enzymol. 546 (2014) 47–78.

[188] S. Mettananda, C.A. Fisher, D. Hay, M. Badat, L. Quek, K. Clark, P. Hublitz, D. Downes, J. Kerry, M. Gosden, J. Telenius, J.A. Sloane-Stanley, P. Faustino, A. Coelho, J. Doondeea, B. Usukhbayar, P. Sopp, J.A. Sharpe, J.R. Hughes, P. Vyas, R.J. Gibbons, D.R. Higgs, Editing an alpha-globin enhancer in primary human hematopoietic stem cells as a treatment for beta-thalassemia, Nat. Commun. 8 (2017) 424.