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REVIEW ARTICLE

Emerging Therapeutic Approaches for Diamond Blackfan Anemia

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Abstract: Diamond Blackfan Anemia (DBA) is an inherited erythroid aplasia with onset in childhood. Patients carry heterozygous mutations in one of 19 Ribosomal Protein (RP) genes, that lead to defective ribosome biogenesis and function. Standard treatments include steroids or blood transfusions but the only definitive cure is allogeneic Hematopoietic Stem Cell Transplantation (HSCT). Although advances in HSCT have greatly improved the success rate over the last years, the risk of adverse events and mortality is still significant.

Clinical trials employing gene therapy are now in progress for a variety of monogenic diseases and the development of innovative stem cell-based strategies may open new alternatives for DBA treatment as well. In this review, we summarize the most recent progress toward the implementation of new therapeutic approaches for this disorder. We present different DNA- and RNA-based technologies as well as new candidate pharmacological treatments and discuss their relevance and potential applicability for the cure of DBA.

Keywords: Bone marrow failure syndrome, diamond blackfan anemia, gene editing, gene therapy, ribosomal protein, ribosomopathy.

1. INTRODUCTION

Diamond-Blackfan Anemia (DBA, OMIM 105650) is a rare macrocytic normochromic anemia usually diagnosed in early infancy and characterized by the selective deficiency of erythroid progenitors in the Bone Marrow (BM). Besides hematological aspects, several physical anomalies have been described in about 50% of patients [1-3]. Like other BM failure disorders, DBA is also a cancer predisposition syndrome and both hematological malignancies and solid tumors have been described in patients with DBA [4, 5].

DBA is characterized by autosomal dominant inheritance with incomplete penetrance and variable expressivity even in the same family. The first DBA gene identified was Ribosomal Protein (*RP*) *S19*, that is mutated in 25% of cases [6, 7]. Heterozygous mutations or single copy deletions have been subsequently detected in other 18 RP genes either of the small (*RPS7*, *RPS10*, *RPS15A*, *RPS17*, *RPS24*, *RPS26*, *RPS27*, *RPS28*, *RPS29*) or of the large (*RPL5*, *RPL11*, *RPL15*, *RPL18*, *RPL26*, *RPL27*, *RPL31*, *RPL35*, *RPL35A*) ribosomal subunit [7-20]. Rare cases of pathogenic mutations in the transcription factor GATA-1 [21, 22] and in the RPS26-interacting protein TSR2 [10] have also been reported in patients with DBA. The genetic cause of DBA remains unknown in approximately one-third of patients. Haploinsufficiency of an RP leads to defective ribosome biogenesis resulting in apoptosis and reduced proliferation of erythroid progenitors through activation of p53–dependent and independent pathways [17, 23, 24]. Several hypotheses have been suggested to explain the selective impairment of erythropoiesis: erythroid progenitors might be particularly prone to apoptosis during ribosomal stress because of p53 stabilization [23] or accumulation of a toxic level of heme [25, 26]. Another possibility is that translation of specific erythroid transcripts is impaired [27].

The first-line treatment is represented by steroids which have various side effects from long-term use. About 50% of patients are unresponsive to steroids and are treated with chronic transfusions with iron chelation to avoid secondary hemochromatosis [28]. A widely employed alternative to life-long transfusions is allogeneic HSCT, but the implementation of alternative therapies is advisable to develop a definitive cure for DBA.

In recent years, tremendous strides have been made in the field of stem cell and genetic therapies. Here we evaluate the potential applicability of these strategies for the cure of DBA, including the employment of innovative DNA- and RNA-based therapies and pharmacological treatments.

2. ADVANTAGES AND LIMITATIONS OF HSCT

At present, HSCT is the only curative option for the hematological manifestations of DBA and it may be proposed to steroid-refractory patients to prevent iron overload due to red cell transfusions. DBA patients appear to be more sus-

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ceptible than other chronically transfused patients to iron overload, which can cause organ toxicity [28, 29], therefore HSCT can be recommended for heavily transfused patients at a very young age. The rate of success of HSCT is high when it is performed on patients younger than 10 years of age from an HLA-identical donor [1, 30]. Some studies described a more positive outcome for HSCT from HLA-matched siblings [1, 31], but the risk of using silent carriers as donors should be considered when the mutated gene has not been identified. Standard conditioning protocol for DBA avoids total body irradiation, since DBA is associated with an increased risk of cancer, but includes busulfan-based myeloablative conditioning, also on patients with no sign of myelodysplastic evolution [30, 32, 33]. Reduced-intensity conditioning regimens have been successfully used in a small number of patients with DBA to minimize the risk of post-transplant neoplasms [34-39].

Notwithstanding the improvements achieved in recent years, HSCT is still associated with significant mortality and morbidity due to infections or Graft *versus* Host Disease (GvHD), especially for patients transplanted from HLA-haploidentical donors. Noteworthy, the successful engraftment of Hematopoietic Stem Cells (HSCs) ensures the resolution of anemia and suggests the potential efficacy of gene therapy on HSCs for DBA treatment.

3. DISEASE CORRECTION BY DNA-BASED STRATEGIES

Gene therapy may be defined as the introduction of genetic material into patient cells for treatment purposes. Typically, a wild-type version of the gene of interest is delivered to target cells to overcome their intrinsic genetic defect. Decades of research in this field have shown how promising, but also challenging, gene therapy can be. Recent advances in the development of viral vector systems have improved the safety of gene transfer and led to major clinical successes [40]. In particular, important results have been achieved by *ex vivo* gene transfer into HSCs for the treatment of hematological and neurodegenerative disorders [41]. It is well known that HSCs have a great therapeutic potential due to their self-renewal capacity. Two clinical trials in Wiskott-Aldrich syndrome and metachromatic leukodystrophy showed remarkable clinical benefits after *ex vivo* gene therapy of HSCs [42, 43]. Considerable progress has been also achieved in the gene therapy of β-thalassemia and sickle cell disease [44, 45]. Presently three clinical trials are recruiting patients with Fanconi Anemia (FA) to evaluate the safety and efficacy of HSC therapy with Lentiviral Vectors (LVs) carrying the *FANCA* gene (ClinicalTrials.gov Identifiers: NCT03157804, NCT03351868, NCT01331018). This demonstrates that the translation from bench to clinic of gene therapy for BM failure syndromes is feasible.

The use of viral vectors ensures high efficiency of gene delivery but has some important drawbacks. Adenoviral Vectors (AdVs) are the most frequently used vectors in clinical trials but pre-existing immunity against them is very common in the general population [46]. Moreover, the transgene is not integrated into the host genome and its expression is diluted over time because of cell proliferation, but multiple infusions of AdVs are not recommended due to their high immunogenicity. Adeno-Associated Viral Vectors (AAVs) are a safer alternative as they show low immunogenicity and the ability to integrate into a specific site in the long arm of chromosome 19, but their transgene capacity is limited to about 4.5 kb [47]. Retroviral and lentiviral vectors (RVs, LVs) lead to the integration of the transgene into the host genome, which allows stable transgene expression also in proliferating tissues, but presents the risk of insertional mutagenesis, that is the inactivation of tumor suppressor genes or the activation of proto-oncogenes, events that may induce carcinogenesis [48, 49]. The deleterious effects due to the unpredictable integration of transgenes constitute a major concern for the employment of RVs and LVs for clinical applications, especially because in the past some young patients who underwent gene therapy for SCID-X1 with gamma-RVs developed leukemia [50, 51]. However, in a different clinical trial for ADA-SCID, no insertional mutagenesis has been reported. This trial, that has been ongoing for over 10 years with a median follow-up of 8 years, uses gamma-RVs that seem safer than those of previous trials, and is important to investigate if the transgene included in the RVs can have an influence in the development of potential adverse effects [52]. Studies on the integration profiles of LVs did not show an overrepresentation of oncogenic sites, and so far no patient treated with LVs-transduced HSCs has developed clonal expansion or leukemic transformation, therefore LVs are considered safer than RVs [42, 53], although extended periods of follow-up are required to establish long-term safety.

3.1. Gene Therapy in DBA Cell and Animal Models

Theoretically, DBA should be an ideal target for therapeutic gene transfer to HSCs, since it is a hematopoietic disease in which corrected cells acquire a selective proliferative advantage [54]. The recent observation of the effects of somatic reversion in a DBA patient represents a good example of how a blood cell population can expand and lead to clinical improvement after the correction of the causal mutation [55]. This patient carried a germline *de novo* deletion including two RP genes on the maternal allele and was transfusion dependent during the first years of life until he underwent remission, a phenomenon observed in about 20% of DBA cases [56]. The mechanisms underlying remission are still poorly understood. The authors suggested that spontaneous recovery in this patient was due to the existence of two different clones in the blood where the maternal chromosome was lost and replaced by a second copy of the wild-type paternal allele [55].

Gene therapy would be able to cure DBA without the need of an HLA-matched donor and of prolonged immunosuppressive therapy. The risk of GvHD would also be abolished and the preconditioning regimen could be reduced or even absent because of the proliferative advantage of the gene-corrected HSCs.

In the past, several studies tried to assess the feasibility of gene therapy in DBA by enforced expression of *RPS19*, the gene most frequently mutated in DBA, in *RPS19*-deficient cells. This approach rescued the pathological phenotype of RPS19-mutated lymphoblastoid cell lines derived from patients, characterized by defects in rRNA maturation, proliferation and protein synthesis, as well as by abnormal p53

activation [57]. The same strategy used for RPL5-haploinsufficient cells achieved only a partial rescue, suggesting that specific investigation will be needed for each DBA gene; this further adds complexity to the development of gene therapies for DBA [57]. Hamaguchi *et al.* reported that transfer of *RPS19* cDNA using oncoretroviral or LVs into RPS19-mutated $CD34^+$ cells isolated from patients with DBA promoted the formation of erythroid colonies both in solid and liquid cultures [58, 59]. Moreover, Flygare *et al.* used such corrected $CD34^+$ cells to transplant sub-lethally irradiated mice and demonstrated that a high level of RPS19 expression conferred a survival advantage to transplanted cells and favored engraftment [54].

The use of animal models for DBA is crucial to investigate the feasibility, the therapeutic efficacy and the safety of gene therapy. The first mouse model able to recapitulate the hematological phenotype of DBA was obtained using transgenic RNA interference that allowed a doxycycline-inducible downregulation of RPS19 [60]. This model developed macrocytic anemia and BM failure that were recovered *in vitro* and *in vivo* by *RPS19* gene transfer using LVs [60, 61]. A subsequent study demonstrated that anemia in these mice was also cured by expression of *RPS19* driven by the elongation factor 1α short promoter, a clinically relevant cellular promoter derived from human genes which may have reduced risk of insertional mutagenesis [62, 63].

The breakthrough of reprogramming mature cells to pluripotency represents a revolution towards personalized therapy because the risk of immune rejection and the ethical concerns of using embryonic cells are eluded. Induced Pluripotent Stem Cells (iPSCs), are an unlimited source of autologous cells that can be genetically manipulated, differentiated into specialized cells and entirely characterized before transplant. Garçon *et al.* obtained iPSCs from skin fibroblasts of two patients with DBA who carried mutations in *RPL5* or *RPS19*, thus providing for the first time a renewable reservoir of cells that display ribosomal and hematopoietic defects [64]. DBA fibroblasts generated iPSC colonies at a frequency of 0.0045%, whereas the efficiency for control fibroblasts was 0.03%. Moreover, most DBA clones showed decreased proliferation and only one stable clone for each genotype could be established. The authors hypothesized that this could be due to the activation of p53 in DBA cells [64]. The ribosomal and hematopoietic abnormalities were recovered *via* DNA transfer of a wild-type copy of the haploinsufficient gene into the "safe harbor" *AAVS1* locus, where integrated transgenes can be stably expressed without the risk of epigenetic silencing or insertional mutagenesis. The proof of principle that these cells completely recapitulate DBA offered the possibility to better understand the pathogenetic mechanisms of the disease. The same investigators performed a transcriptome analysis of DBA iPSCs and observed the dysregulation of the Transforming Growth Factor β (TGFβ) signaling pathway [65]. They also carried out a drug screen to discover molecules able to stimulate erythropoiesis in this cell model and identified SMER28, an inducer of autophagy, as a candidate therapeutic agent [66].

The possibility to genetically correct DBA iPSCs and employ them to regenerate the defective tissue is attractive, but it has to be considered that reprogramming of DBA fibroblasts to iPSCs had a very low efficiency and this limits the future applications of this strategy. Moreover, skin fibroblasts, especially those derived from adult patients, may show a high burden of somatic mutations due to UV exposition [67]. As a matter of fact, the detection of copy-number alterations in fibroblast-derived iPSCs was one of the reasons for the premature conclusion of the first clinical trial that used iPSCs to cure macular degeneration [68]. More in general, twelve years after Yamanaka's discovery [69], iPSCs have made their mark in human disease modeling, but the implementation of iPSC-based therapies proved to be very challenging. Nevertheless, DBA iPSCs represent a fundamental tool to investigate the molecular mechanisms underlying this disorder after the definition of the best cellular target to be reprogrammed with a consistent efficiency. Along with editing technologies, these cells may be useful to study the effect of specific mutations and translate the consequent findings to a personalized medicine.

3.2. *Ex Vivo versus In Vivo* Gene Therapy

Ex vivo gene transfer is directed to the cells of interest (*e.g.* HSC) before their reinjection into the patient and therefore acts selectively on target cells preventing both the transduction of cells that would not benefit from the genetic modification and the activation of immune responses. The BM of DBA patients is frequently normocellular with selective erythroid hypoplasia, therefore the collection of an adequate number of HSCs for *ex vivo* therapy, although probably not as efficient as for other disorders, is expected to be easier than in FA, where the accumulation of mutations secondary to the abnormal DNA repair system leads to progressive loss of stem cells.

In vivo gene transfer is an alternative method for gene delivery that avoids some of the drawbacks of *ex vivo* transfer, in particular, the need to collect a sufficient number of HSCs from BM or peripheral blood, and to manipulate them in *ex vivo* cultures. With this technique the viral vectors carrying the therapeutic gene can target HSCs directly in their environment, thus ensuring the maintenance of physiological conditions. Specific promoters or microRNA (miRNA) target sequences can be added to restrict transgene expression to a particular cell type [70, 71]. The intrafemoral infusion of lentiviral particles encoding *FancC* in $FancC^{-/-}$ mice, a model of FA, efficiently corrected the phenotype of HSCs [72], and the intraosseous delivery of LVs encoding factor VIII corrected murine hemophilia A [73]. It would be interesting to evaluate this *in vivo* procedure in the DBA mouse model as well.

3.3. Future Strategies to Correct DBA by Gene Editing

In the last years, the advent of genome-editing technologies has overturned the field of gene therapy. Unlike gene addition, gene editing avoids the risk of insertional mutagenesis because it precisely targets the affected gene restoring its function and maintaining its endogenous expression regulation. This technology allows the achievement of therapeutic effect by correction of disease-causing mutations or removal of deleterious genome sequences [74]. A turning point for gene editing was the discovery that introduction of site-specific Double-Strand Breaks (DSB) in the human genome

stimulates the endogenous repair machinery. The repair by non homologous end-joining (NHEJ) often causes insertions or deletions and disruption of gene function, whereas the repair by homology direct repair (HDR) can lead to precise gene correction if a wild-type template is provided.

The three most commonly used genome editing technologies are Zinc Finger Nucleases (ZFNs), Transcription Activator Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated Cas9 (CRISPR/Cas9). Both ZFNs and TALENs consist of a specific DNA-binding domain and a non-specific endonuclease domain of the FokI restriction enzyme. Compared with ZFNs, TALENs are easier to design, have higher targeting flexibility and efficiency and show a reduced off-target activity. Mutations in the β-globin gene (*HBB*) in iPSCs derived from patients with β-thalassemia or sickle cell disease have been corrected using TALENs [75, 76]. This technology has also been applied to target *RUNX1* in iPSCs from familial platelet disorder with propensity to acute myeloid leukemia [77]. Disadvantages of TALENs are the large size that requires a vector with appropriate packaging capacity, and the presence of repetitive sequences. The unstable nature of these repeats can induce rearrangements when TALENs are delivered by LVs [78].

The most recent and promising genome editing tool is CRISPR/Cas9. Instead of engineered proteins, this system exploits a guide RNA (gRNA) designed to hybridize with a specific genomic site where the Cas9 enzyme will create a DSB with high efficiency. The simple modification of the short gRNA sequence allows targeting any gene of interest; therefore the applications of the CRISPR/Cas9 system have increased exponentially. Neither CRISPR/Cas9 nor TALENs have perfect DNA recognition specificity and unwanted sequence changes can occur in other sites of the genome, with unpredictable consequences for the cell. The development of reliable methods to anticipate and reduce these off-target effects is in progress. However, the use of CRISPR/Cas9 is much more rapid, simple and efficient than TALENs and ZFNs. Several research groups have successfully applied CRISPR-Cas9 technology to correct β-thalassemia mutations in patient-derived iPSCs [79-81]. Similarly, primary fibroblasts and iPSCs from patients with FA have been recovered using CRISPR-Cas9 [82, 83]. This disease is characterized by deficiencies in the DNA repair system, the same machinery required for genome editing. Since DNA repair is not defective in DBA cells, it is likely that CRISPR/Cas9 technology may be more efficient in DBA than in FA.

Altogether, these findings suggest that CRISPR/Cas9 represents the most rapid and reliable editing technology for DBA research, even though, at present, no experimental evidence of the feasibility of gene editing in DBA is available yet.

4. DISEASE CORRECTION BY RNA-BASED STRATEGIES

Among the emerging solutions for the therapy of currently incurable genetic diseases, RNA-targeting strategies hold the potential for specific gene expression modulation. In August 2018 the first therapy based on Small Interfering RNA (siRNA) was approved by the Food and Drug Administration (FDA). This therapy aimed to silence the expression of transthyretin in hereditary transthyretin amyloidosis [84, 85]. RNA molecules such as siRNAs, miRNAs and aptamers, a class of oligonucleotides that behave like "chemical antibodies" [86], cannot be beneficial in DBA, but other RNA-based treatments effective for DBA patients might be developed in the future, as long as some key issues, including instability, insufficient delivery to target cells, immunogenicity, and off-target toxicity, are addressed [87].

4.1. Messenger RNA Reprogramming by Spliceosome-Mediated RNA Trans-Splicing (SMaRT)

The technology of Spliceosome-Mediated RNA Trans-splicing (SMaRT) can modify a target mRNA sequence at the post-transcriptional level. SMaRT exploits the ability of the spliceosome to carry out trans-splicing between two different RNA molecules: the mutated endogenous transcript and a synthetic RNA delivered into the cell by gene transfer. The resulting product is a chimeric mRNA encoding a sequence without mutations [88]. The most important added value of this technology is the conversion of mutant transcripts into wild-type mRNAs for the correction of disorders due to dominant negative mutations [88]. Based on data obtained in a mouse model, a dominant negative mechanism was proposed to explain the effect of an *RPS19* missense mutation identified in a small number of patients with DBA [89]. Although not well established in DBA, this possible pathogenetic mechanism has to be taken into account. However, the trans-splicing process needs to be better investigated.

4.2. Enhancing Translation of Target mRNAs by SINEUPs

Another RNA-based technology potentially useful for therapeutic purposes is represented by SINEUPs, a functional class of long non-coding antisense RNAs that can increase the translation of a specific transcript by partially overlapping the 5' UTR of the target mRNA [90]. The antisense sequence in synthetic SINEUPs can be designed to enhance expression of any gene of interest, with the advantage that the upregulation induced by SINEUPs is within a physiological range (approximately 2 fold), avoiding possible side effects due to overexpression. Overexpression of some RPs such as RPL5 and RPL11 is expected to be detrimental for the cell because it can activate p53 [91], and SINEUP technology would overcome this issue. SINEUPs have been used to rescue the pathological phenotype in a medaka fish model of microphthalmia with linear skin defects syndrome [92]. However, the mechanism of action of SINEUPs has not been sufficiently elucidated and further studies are required to understand whether this tool can upregulate effectively the translation of RP transcripts, and how the presence of missense mutations could affect SINEUP function.

4.3. Upregulation of Gene Expression Using Small Activating RNAs

Small Activating RNA (saRNAs) are a class of RNA molecules able to activate the expression of a target gene by binding its promoter region, a phenomenon called RNA

Table 1. Comparison of the advantages and disadvantages of HSCT to future possible DNA- and RNA-based therapeutic approaches for DBA.

Present Therapeutic Strategies		Advantages	Disadvantages	Refs.
Hematopoietic stem cell transplantation		• One treatment is resolutive, if successful • High rate of success for HLA-matched donors • Feasible without knowing the causative mutation	• Risk of GvHD • Adverse effects due to preconditioning • Risk of unknown mutations in silent carriers • Immunosuppressive therapy	[1, 30, 31]
Future therapeutic strategies		**Advantages**	**Disadvantages**	**Refs.**
DNA-based	*Ex vivo* gene addition in HSCs	• One treatment is resolutive, if successful • No need for a donor; no need for prolonged immunosuppressive therapy; no GvHD occurrence • Reduced or absent preconditioning	• The causative mutation must be known • Off targets effects • Risk of insertional mutagenesis	[54, 57-59, 61]
	Ex vivo gene addition in iPSCs	• One treatment is resolutive, if successful • No need for a donor; no need for prolonged immunosuppressive therapy; no GvHD occurrence • Unlimited source of autologous cells • Cell genome can be studied before reinfusion • Reduced or absent preconditioning	• The causative mutation must be known • Very low reprogramming efficiency • Risks connected to reprogramming procedure • Off targets effects • Risk of insertional mutagenesis • Risk of somatic mutations in the cells of origin	[64, 65, 68, 69]
	In vivo gene addition	• One treatment is resolutive, if successful • No need for a donor; no need for prolonged immunosuppressive therapy; no GvHD occurrence • No preconditioning	• The causative mutation must be known • Off targets effects • Risk of insertional mutagenesis • Possible immune response against the vector • Lack of data for the application of this technology to RP genes	[71-73]
	Gene editing	• One treatment is resolutive, if successful • No need for a donor; no need for prolonged immunosuppressive therapy; no GvHD occurrence • No preconditioning • Gene expression is under the regulation of endogenous mechanisms	• The causative mutation must be known • Off targets effects • Lack of data for the application of this technology to RP genes	[74, 82]
RNA-based	SMaRT SINEUPs saRNAs	• No need for a donor; no need for prolonged immunosuppressive therapy; no GvHD occurrence • No preconditioning • Theoretically very specific • The expression of the deficient gene is increased to a physiological level	• Chronic administrations are needed • The causative mutation must be known • Off targets effects • Lack of data for the application of these technologies to RP genes	[88, 90, 92-94, 96, 97]

activation (RNAa). SaRNAs are double-stranded, 19-21 nucleotides long molecules that were first discovered by investigators studying the role of small RNAs in gene silencing [93, 94]. The exact molecular mechanisms of RNAa mediated by saRNAs have not been elucidated, but it is known that saRNAs can associate to the protein Argonaute (Ago) 2, forming a nucleoprotein complex called RNA-induced transcriptional activation (RITA) complex. The RITA complex recognizes complementary sequences on the promoter of the target gene and induces histone modification and transcription initiation [95]. The modulation of transcriptional activity by saRNAs might be employed not only to study gene function but also for therapeutic applications in various diseases [96, 97]. No data on RNAa of ribosomal protein genes is yet available.

5. NEW DRUGS

Besides the advances in DNA and RNA-based approaches, several pharmacological treatments have been pro-

posed for the management of DBA in the last few years. The development of effective drugs is especially critical for those patients who are not eligible for HSCT or gene therapy, because, for example, no HLA-matched donor is available or the affected gene is unknown.

Using erythroid progenitors purified from mouse fetal liver, Flygare and coll. demonstrated that Glucocorticoids (GC) increase the production of erythroid cells by inducing Burst Forming Units-Erythroid (BFU-E) self-renewal [98]. Some of the transcriptional targets of the GC dexamethasone (Dex) were also upregulated by prolyl hydroxylase inhibitors (PHIs), drugs that are being tested to treat the anemia secondary to chronic kidney disease [98]. *In vitro* culture experiments showed that the addition of the PHI dimethyloxalylglycine, together with Dex, resulted in a synergistic increase of BFU-E proliferation and self-renewal [98]. One of the genes whose expression is induced by Dex is the peroxisome Proliferator-Activated Receptor α (PPAR-α) [99]. PPAR-α agonists such as GW7647 and fenofibrate have been shown to synergize with GC, promote BFU-E self-renewal and improve red cell production. Interestingly, fenofibrate is a U.S. FDA-approved drug for the treatment of hypercholesterolemia and hypertriglyceridemia. The use of PPAR-α agonists might reduce the dose of GC required to sustain erythropoiesis in steroid-responsive patients with DBA.

The discovery that the TGF-β pathway is dysregulated in DBA iPSCs paved the way to the employment of new drugs that block TGF-β signaling, such as Galunisertib (LY2157299 monohydrate) [65]. This small molecule has been shown to promote red cell production by stimulating self-renewal of BFU-E [100]. Galunisertib is now being evaluated in various clinical trials for its anticancer activity; whether it is a suitable candidate for the treatment of patients with DBA remains to be determined.

Finally, an ongoing clinical trial (ClinicalTrials.gov Identifier: NCT01464164) will assess the efficacy of Sotatercept (ACE-011) in adult patients with DBA. Sotatercept was originally developed and tested as a potential treatment for osteoporosis, but *ad hoc* clinical trials showed that it also positively regulated erythrocyte production [101]. This small molecule acts as an activin receptor type IIA ligand trap and inhibits TGF-β signaling. Its murine orthologous RAP-011 improved erythropoiesis in a DBA zebrafish model, further supporting the use of Sotatercept in patients with DBA [102].

CONCLUDING REMARKS

To date, the only definitive cure for the hematological manifestations of DBA is HSCT, which can cause life-threatening side effects and achieves optimal outcomes only if an HLA-matched donor is available. Here we described several DNA and RNA-based procedures and new pharmacological options whose employment in DBA might be pursued in the near future. Comparison of both the advantages and disadvantages of HSCT to future DNA-based and RNA-based therapeutic approaches for DBA are shown in Table **1**. Further detailed studies are needed to evaluate which strategies are most likely to succeed. Among the different novel strategies described above, the clinical application of gene replacement by *ex vivo* manipulation of patient HSCs with LVs or AAVs seems imminent, at least for *RPS19*, that is the most studied DBA gene in cell and animal models [54, 57, 61]. Corrected cells should gain a proliferative advantage over RP-deficient cells [54, 55]. Such an approach has proved to be feasible and effective for other monogenic diseases, and, together with gene editing, probably represents the most promising approach for a safe and long-term cure of DBA. On the other hand, DBA introduces further challenges compared to most of the currently treated diseases, that are the involvement of many different genes and the fact that for several patients the causative mutation is unknown. Therefore, advancements in drug development and HSCT procedures are critical.

Importantly, the occurrence of spontaneous remission in about 20% of DBA patients implies an apparently unsolvable ethical dilemma in the choice among HSCT, gene therapies and other less risky, but less effective treatments. The validation of these approaches will help to understand the best strategy to develop in future clinical trials for an effective treatment for DBA.

LIST OF ABBREVIATIONS

AAV	=	Adeno-Associated Virus
AdV	=	Adenoviral Vector
BFU-E	=	Burst Forming Units-Erythroid
BM	=	Bone Marrow
CRISPR/Cas9	=	Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-associated Cas9
DBA	=	Diamond Blackfan Anemia
Dex	=	Dexamethasone
DSB	=	Double-Strand Break
FA	=	Fanconi Anemia
FDA	=	Food and Drug Administration
GC	=	Glucocorticoid
gRNA	=	guide RNA
GvHD	=	Graft *versus* Host Disease
HDR	=	Homology Direct Repair
HSC	=	Hematopoietic Stem Cell
HSCT	=	Hematopoietic Stem Cell Transplantation
iPSC	=	induced Pluripotent Stem Cell
LV	=	Lentiviral Vector
miRNA	=	microRNA
NHEJ	=	Non-Homologous End-Joining
PHI	=	Prolyl Hydroxylase Inhibitor
PPAR-α	=	Peroxisome Proliferator-Activated Receptor α
RP	=	Ribosomal Protein
RV	=	Retroviral Vector
saRNA	=	small Activating RNA
SMaRT	=	Spliceosome-Mediated RNA Trans-splicing
TALEN	=	Transcription Activator-Like Effector Nuclease
TGFβ	=	Transforming Growth Factor β
ZFN	=	Zinc Finger Nuclease

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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