Gene therapy using haematopoietic stem and progenitor cells

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Abstract | Haematopoietic stem and progenitor cell (HSPC) gene therapy has emerged as an effective treatment modality for monogenic disorders of the blood system such as primary immunodeficiencies and β-thalassaemia. Medicinal products based on autologous HSPCs corrected using lentiviral and gammaretroviral vectors have now been approved for clinical use, and the site-specific genome modification of HSPCs using gene editing techniques such as CRISPR–Cas9 has shown great clinical promise. Preclinical studies have shown engineered HSPCs could also be used to cross-correct non-haematopoietic cells in neurodegenerative metabolic diseases. Here, we review the most recent advances in HSPC gene therapy and discuss emerging strategies for using HSPC gene therapy for a range of diseases.

Allogeneic
Relating to or denoting that the source of cells, tissues or organs for transplant is from an individual genetically different from the recipient.

Primary immunodeficiencies
(PIDs). Mendelian genetic disorders caused by defects in the development and/or function of immune cells. Currently, more than 300 genes have been identified that cause adaptive and/or innate immune cell defects.

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Haematopoietic stem cell (HSC) transplantation (HSCT) has been a routine procedure for treating inborn errors of metabolism and the blood system for more than 50 years[1,2]. The first successful transplantations for the treatment of immune disorders were conducted in 1968 using allogeneic stem cells to treat X-linked severe combined immunodeficiency (SCID-X1), an inherited disease caused by inactivating mutations in the gene encoding interleukin-2 receptor subunit-γ (*IL2RG*), and Wiskott–Aldrich syndrome, a rare X-linked recessive immunodeficiency characterized by thrombocytopenia, eczema and recurrent infections[3,4]. Extraordinary progress has been made in allogeneic HSCT, and it is now used for many genetic diseases in an increasing number of patients. Improvements have been made with regard to donor matching, strategies for more effective control of graft-versus-host disease, more effective conditioning regimens and better management of toxicity and infections. However, the availability of immunocompatible donors with optimal human leukocyte antigen (HLA) genotype matching can limit its application[5], and morbidity owing to graft-versus-host disease remains as a result of the use of unmatched donors. As a result, gene therapy techniques based on the genetic modification of autologous haematopoietic stem and progenitor cells (HSPCs) have been explored.

Autologous HSPC gene therapy has been investigated for the prevention or treatment of monogenic disorders associated with altered blood cell maturation and function, such as diseases of the innate and adaptive immune systems, red blood cell disorders, platelet disorders and bone marrow failure syndromes[6–8]. HSPC gene therapy techniques to date have employed ex vivo gene transfer, through transduction of the patients' own HSPCs with a vector carrying one or more copies of a therapeutic gene[7]. Once reinfused, genetically modified HSPCs undergo self-renewal and establish a population of modified cells, which pass the transgene to daughter blood cells on differentiation (FIG. 1). The first proof-of-concept HSPC gene therapy studies were conducted in the 1990s to address adenosine deaminase (ADA) deficiency SCID (ADA-SCID); although correction was successful, the efficiency of correction in these studies was low[9–12]. Improvements in ex vivo culture techniques and new engineering approaches using long terminal repeat (LTR)-driven gammaretroviral vectors have since resulted in clinical benefit in many primary immunodeficiencies (PIDs)[13,14]; however, the occurrence of T cell acute lymphoblastic leukaemia following gene therapy in a significant proportion of patients — caused by insertional mutagenesis into the *LMO2* locus — led to a substantial slowdown of all ex vivo gene therapy approaches[15,16]. The development of self-inactivating lentiviral vectors as a delivery platform[7] has since enabled safer and more effective insertion of therapeutic genes into HSPCs.

Over the past two decades, advances in our understanding of disease biology and gene transfer technology have been translated into remarkable clinical successes (FIG. 2). As a result, the field has attracted significant commercial interest, and two products have secured regulatory approval by the European Medicines Agency (EMA) in Europe — namely Strimvelis for use in ADA-SCID and Zynteglo for use in patients older than 12 years with transfusion-dependent β-thalassaemia — with several others expected to follow in both Europe and the United States in the next 5 years. More than 300 patients have now been treated with HSPC gene therapy in clinical trials, and robust evidence for the durability of corrected HSPC treatments and their long-term safety and

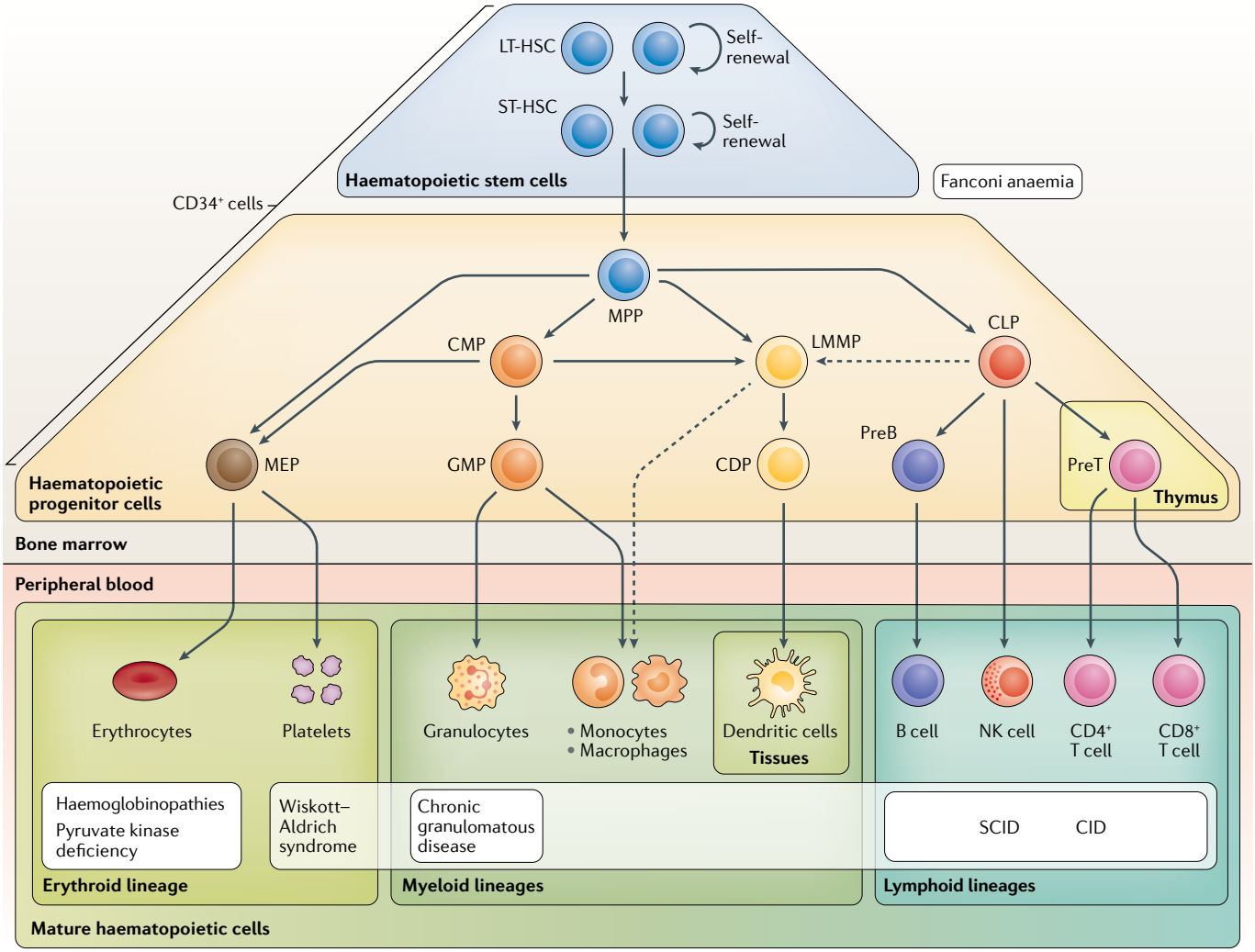

Fig. 1 | **The haematopoietic hierarchy and genetic disorders.** Bone marrow-resident haematopoietic stem cells and haematopoietic progenitor cells replenish blood and tissues with new mature cells. Both haematopoietic stem cells and haematopoietic progenitor cells express the cell surface marker CD34, which is used to enrich a mixture of haematopoietic stem and progenitor cells for transplantation and gene therapy. Haematopoietic stem cells can be classed as long-term haematopoietic stem cells (LT-HSCs) or short-term haematopoietic stem cells (ST-HSCs). ST-HSCs progressively acquire lineage specifications in order to differentiate into lineage-committed progenitors and eventually terminally differentiated cells, which are released into the peripheral blood. A simplified scheme of human haematopoiesis is presented here. Alternative models have been postulated on the basis of cell surface marker analyses, in vitro and in vivo functional assays, clonal tracking by insertion analyses in haematopoietic stem and progenitor cell gene therapy studies and single-cell RNA analyses (reviewed previously[22]). Mendelian genetic disorders can affect self-renewal, differentiation and/or the function of different blood and immune cells. Examples of genetic diseases for which gene therapy is under investigation or approved are represented in white boxes below affected cell types. Wiskott–Aldrich syndrome affects platelets and other lineages. CDP, common dendritic progenitor; CID, combined immunodeficiency; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulomonocytic progenitor; LMMP, lymphoid-myeloid primed progenitor; MEP, megakaryocytic–erythroid progenitor; MPP, multipotent progenitor; NK cell, natural killer cell; preB, pre-B cell; preT, pre-T cell; SCID; severe combined immunodeficiency.

clinical efficacy has been obtained for PIDs, including SCID-X1, ADA-SCID, Wiskott–Aldrich syndrome, chronic granulomatous disease (CGD), β-thalassaemia, metachromatic leukodystrophy (MLD) and X-linked adrenoleukodystrophy (X-ALD) (TABLE 1).

Here, we review the most recent advances in HSPC gene therapy. We begin with an overview of the ex vivo gene transfer process, from HSPC collection and genetic modification to engraftment and long-term clonal tracking. We discuss recent technological developments in gene editing, which will help move the field forward to the next generation of medicinal products, broadening the field of application to disorders not amenable to current gene addition approaches. Finally, we discuss the application of HSPC gene therapy for different diseases, including the use of HSPCs as delivery vehicles for therapeutic proteins, and conclude by considering remaining challenges in the field and future perspectives.

Chronic granulomatous disease
(CGD). A disease caused by dysfunction of the phagocyte NADPH oxidase, a membrane-bound enzyme complex required for effective killing of bacteria and fungi.

Ex vivo gene transfer

HSPC collection

Autologous HSPCs are collected either through multiple aspirations from the iliac crests or leukapheresis following the administration of mobilizing agents (FIG. 3), and collected material is enriched for CD34⁺ cells. Both procedures yield a mixture of non-engrafting

Leukapheresis
A procedure that separates white blood cells, including haematopoietic stem cells, from the blood. White cells are collected from the donor and other blood fractions are returned to the circulation.

Mobilizing agents
Drugs that induce transient mobilization of haematopoietic stem cells from the bone marrow to the circulation so that they can be collected by leukapheresis.

cells and a heterogenous population of primitive and lineage-committed HSPCs that includes short-term repopulating haematopoietic progenitor cells (HPCs) contributing to early reconstitution and a very small fraction of long-term repopulating HSCs that take longer to restore haematopoiesis (FIG. 1). The relative composition of HSCs and HPCs in the collected material depends on disease background, age and the collection method and mobilization procedure used[17–20]. The yield of $CD34^+$ cells following bone marrow aspiration negatively correlates with donor weight and age[21]. Preliminary results of clinical trials for immunodeficiencies such as Wiskott–Aldrich syndrome indicate that both mobilized peripheral blood and bone marrow sources engraft and lead to clinical benefit[22], although in-depth comparative studies between the two sources are lacking. However, as leukapheresis provides more HSPCs than bone marrow harvest per donor and allows faster haematopoietic reconstitution[23], it is now the preferred procedure. Granulocyte colony-stimulating factor was the first mobilizing agent used for allogeneic transplantation; however, as some patients have a limited mobilization response to granulocyte colony-stimulating factor[23], it is usually given in combination with the CXCR4 antagonist plerixafor in the context of gene therapy[22,24–27]. Administration of plerixafor alone has been shown to enrich HSPCs with primitive and repopulating features — albeit with a lower yield than when used in combination with granulocyte colony-stimulating factor[19]. Some studies have shown the feasibility of purifying primitive

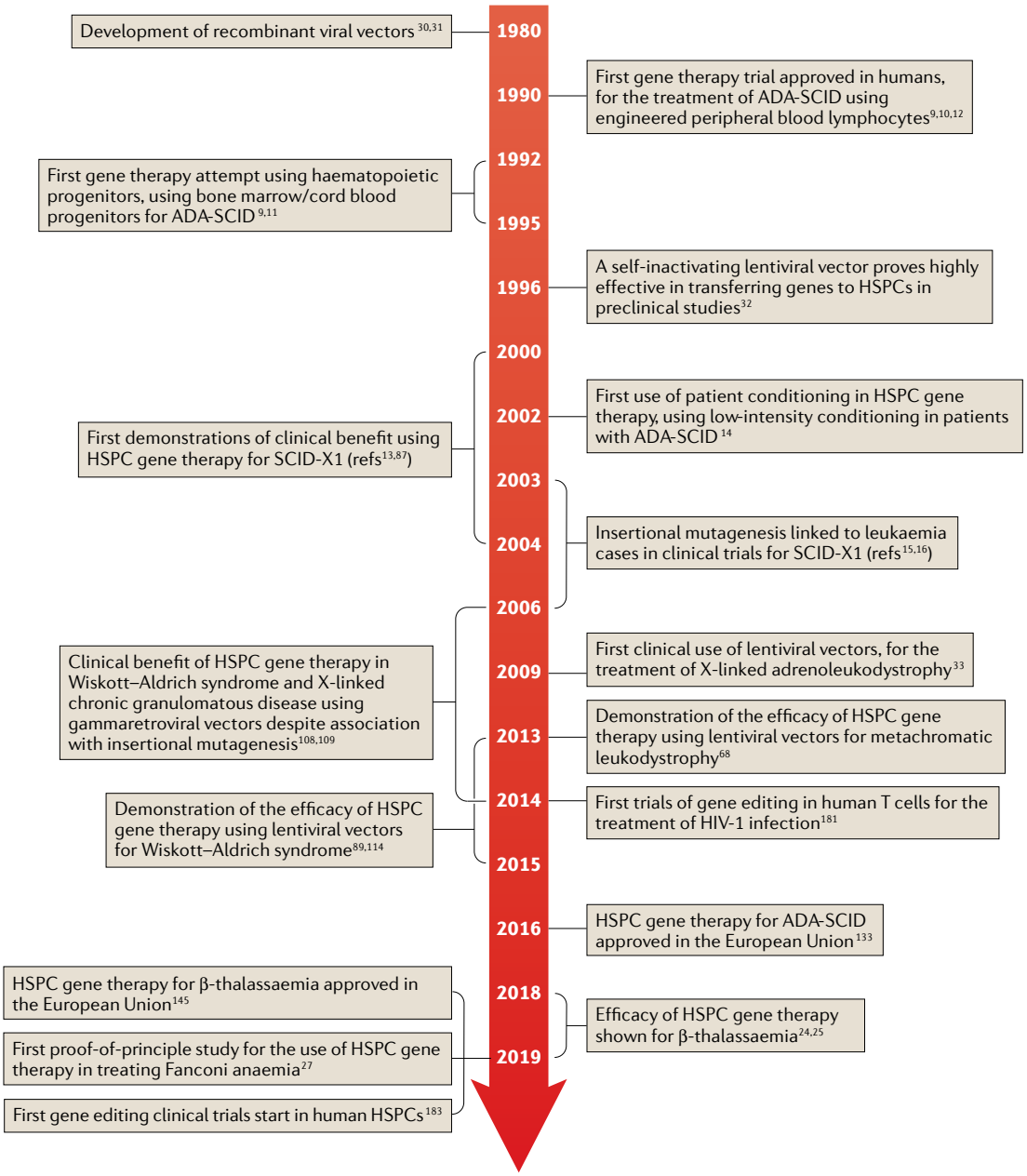

Fig. 2 | **A timeline of HSPC gene therapy.** Progress of haematopoietic stem and progenitor cell (HSPC) gene therapy for use in monogenic disorders and associated enabling technological developments. ADA-SCID, adenosine deaminase deficiency severe combined immunodeficiency; SCID-X1, X-linked severe combined immunodeficiency.

Table 1 | **Ongoing clinical trials for monogenic disorders using ex vivo HSPC gene therapy**

Clinical trial registry numbers (trial phase)	Disease (defective gene)	Conditioning intensity and chemotherapy	Corrective strategy
NCT03311503 (I/II), NCT03601286 (I/II)	SCID-X1 (*IL2RG*)	Low-dose busulfan	Autologous CD34$^+$ cells transduced with the G2SCID lentiviral vector
NCT03217617 (I/II)	SCID-X1 (*IL2RG*)	Not known	Autologous CD34$^+$ cells transduced with the self-inactivating lentiviral vector TYF-IL-2Rg
NCT01306019 (I/II)	SCID-X1 (*IL2RG*)	Low-dose busulfan	Autologous CD34$^+$ cells transduced with the VSV-G pseudotyped lentiviral CL20-4i-EF1a-hyc-OPT vector
NCT02999984 (I/II), NCT03765632 (I/II), NCT04140539 (II/III)	ADA-SCID (*ADA*)	Low-dose busulfan	Cryopreserved autologous CD34$^+$ cells treated with an EFS-ADA lentiviral vector (OTL-101)
NCT03645460 (NA)	ADA-SCID (*ADA*)	Not known	Autologous CD34$^+$ cells transduced with the improved self-inactivating lentiviral vector TYF-ADA
NCT01515462 (I/II) NCT03837483 (II)	WAS (*WAS*)	Reduced intensity (busulfan and fludarabine)	Autologous CD34$^+$ haematopoietic stem cells transduced ex vivo with a lentiviral vector carrying *WAS* (OTL-103)
NCT01410825 (I/II), NCT02333760 (I/II)	WAS (*WAS*)	Myeloablative (busulfan and fludarabine)	Autologous CD34$^+$ haematopoietic stem cells transduced with the lentiviral vector w1.6W
NCT01347346 (I/II), NCT01347242 (I/II)	WAS (*WAS*)	Myeloablative (busulfan and fludarabine)	Autologous CD34$^+$ cells transduced with a lentiviral vector carrying *WAS*
NCT01855685 (I/II), NCT02234934 (I/II)	X-CGD (*CYBB*)	Myeloablative (busulfan)	Autologous CD34$^+$ cells transduced with a lentiviral vector carrying human *CYBB* (OTL-102)
NCT02757911 (I/II)	X-CGD (*CYBB*)	Myeloablative (busulfan)	Autologous CD34$^+$ cells transduced with the G1XCGD lentiviral vector
NCT03812263 (I/II), NCT03825783 (I)	LAD (*CD18*)	Myeloablative (busulfan)	Autologous CD34$^+$ cells transduced with the lentiviral vector Chim-CD18-WPRE, which carries the *ITGB2* gene
NCT03538899 (I/II)	ART-SCID (*DCLRE1C*)	Submyeloablative (busulfan)	Autologous CD34$^+$ cells transduced with the self-inactivating lentiviral vector AProArt, carrying the corrected *DCLRE1C* gene
NCT02906202 (III), NCT03207009 (III)	TDT (*HBB*)	Myeloablative (busulfan)	Autologous CD34$^+$ cells transduced with a lentiviral β^{T87}Q globin vector (LentiGlobin BB305 drug product)
NCT02453477 (I/II)	TDT (*HBB*)	Myeloablative (treosulfan and thiotepa)	Autologous CD34$^+$ cells transduced with the GLOBE lentiviral vector (OTL-300)
NCT03655678 (I/II)	TDT (*HBB*)	Myeloablative (busulfan)	Gene editing of the erythroid enhancer of *BCL11A* using CRISPR–Cas9 to increase HbF expression
NCT03432364 (I/II)	TDT (*HBB*)	Myeloablative (busulfan)	Gene editing of the erythroid enhancer of *BCL11A* using zinc-finger nucleases
NCT02151526 (I/II), NCT02140554 (I/II)	SCD (*HBB*)	Myeloablative (busulfan)	Autologous CD34$^+$ cells transduced with a lentiviral β^{T87Q} globin vector (LentiGlobin BB305 drug product)
NCT02186418 (I/II)	SCD (*HBB*)	Reduced intensity (melphalan)	Autologous CD34$^+$ cells transduced with a γ-globin lentiviral vector (ARU-1801)
NCT03964792 (I/II)	SCD (*HBB*)	Not known	Autologous CD34$^+$ cell transduced with the GLOBE1 lentiviral vector, which carries the βAS3 globin gene (DREPAGLOBE)
NCT02247843 (I/II)	SCD (*HBB*)	Myeloablative (busulfan)	Autologous CD34$^+$ cells transduced with the Lenti/G-βAS3-FB lentiviral vector
NCT03282656 (I)	SCD (*HBB*)	Myeloablative (busulfan)	CD34$^+$ cells transduced with a lentiviral vector containing a short-hairpin RNA targeting *BCL11A* to increase HbF expression
NCT04091737 (I)	SCD (*HBB*)	Reduced intensity (melphalan)	Autologous CD34$^+$ cells transduced with a lentiviral vector encoding human γ-globin G16D and a short-hairpin RNA (RNA734) targeting the hypoxanthine guanine phosphoribosyltransferase gene *HPRT* (CSL200)

Table 1 (cont.) | **Ongoing clinical trials for monogenic disorders using ex vivo HSPC gene therapy**

Clinical trial registry numbers (trial phase)	Disease (defective gene)	Conditioning intensity and chemotherapy	Corrective strategy
NCT03745287 (I/II)	SCD (*HBB*)	Myeloablative (busulfan)	Gene editing of the erythroid enhancer of *BCL11A* using CRISPR–Cas9 to increase HbF expression
NCT03157804 (I/II), NCT04069533 (II), NCT01331018 (I), NCT03814408 (I)	Fanconi anaemia type A (*FANCA*)	No conditioning	Autologous CD34$^+$ cells transduced with a lentiviral vector carrying *FANCA*
NCT03351868 (NA)	Fanconi anaemia type A (*FANCA*)	Not known	Autologous haematopoietic stem cells and mesenchymal stem cells transduced with a lentiviral vector carrying *FANCA*
NCT04105166 (I)	PKD (*PKLR*)	Not known	Autologous CD34$^+$ cells transduced with a lentiviral vector containing *PKLR* (RP-L301)
NCT01560182 (I/II), NCT03392987 (II)	MLD (*ARSA*)	Myeloablative (busulfan)	Autologous CD34$^+$ cells transduced with a lentiviral vector encoding human *ARSA* cDNA (OTL-200, Libmeldy)
NCT01896102 (II/III)	X-ALD (*ABCD1*)	Myeloablative (busulfan and cyclophosphamide)	Autologous CD34$^+$ cells transduced with the self-inactivating lentiviral vector MNDprom-ABCD1 Lenti-D
NCT03852498 (III)	X-ALD (*ABCD1*)	Myeloablative (busulfan and fludarabine)	Autologous CD34$^+$ cells transduced with the self-inactivating lentiviral vector MNDprom-ABCD1 Lenti-D
NCT03488394 (I/II)	MPSI (*IDUA*)	Myeloablative (busulfan and fludarabine)	Autologous CD34$^+$ cells transduced with a lentiviral vector carrying human *IDUA* (OTL-203)
NCT04201405 (I/II)	MPSIIIA (*SGSH*)	Myeloablative (busulfan)	Autologous CD34$^+$ cells transduced with a lentiviral vector carrying human *SGSH*
NCT03897361 (I/II)	Cystinosis (*CTNS*)	Myeloablative (busulfan)	Autologous CD34$^+$ cells transduced with a pCCL-CTNS lentiviral vector carrying the human *CTNS* cDNA sequence
NCT02800070 (I), NCT03454893 (I/II)	Fabry disease (*GLA*)	Myeloablative	Autologous CD34$^+$ cells transduced with the lentivirus vector AVR-RD-01 carrying *GLA*

Data taken from a search performed on April 2020 at https://www.clinicaltrials.gov for currently active (recruiting or not recruiting) studies based on the search terms ['haematopoietic stem cell' OR 'gene therapy'] AND ['gene editing' and 'genetic diseases']. Studies for which information has not been updated in the past 2 years have not been considered. ADA-SCID, adenosine deaminase deficiency severe combined immunodeficiency; ART-SCID, Artemis-deficiency severe combined immunodeficiency; HbF, fetal haemoglobin; HSPC, haematopoietic stem and progenitor cell; LAD, leukocyte adhesion deficiency; MLD, metachromatic leukodystrophy; MPSI, mucopolysaccharidosis type I; MPSIIIA, mucopolysaccharidosis type IIIA; NA, not available (trials without FDA-defined phases); PKD, pyruvate kinase deficiency; SCD, sickle cell disease; SCID-X1, X-linked severe combined immunodeficiency; TDT, transfusion-dependent β-thalassaemia; X-ALD, X-linked adrenoleukodystrophy; X-CGD, X-linked chronic granulomatous disease; WAS, Wiskott–Aldrich syndrome.

HSPCs[28,29] for use as a starting population for ex vivo gene therapy; however, it is likely that primitive HSPCs will take longer than more committed HPCs for reconstitution, and should therefore be combined with HPCs for timely engraftment.

Vector-based modification of HSPCs

Following HSPC collection and enrichment, CD34$^+$ cells are subject to gene transfer. Most gene transfer approaches to date have used viral vectors such as gammaretroviruses and lentiviruses to integrate a therapeutic gene into the genome of the recipient cell (FIG. 4a). In the early 1990s, Moloney murine leukaemia virus-derived gammaretroviral vectors were the first vectors used for HSPC gene therapy for immunodeficiencies, including ADA-SCID[30,31], and were followed in 2006 by HIV-derived, self-inactivating lentiviral vectors[32] for X-ALD[33] and in 2010 by self-inactivating gammaretroviral vectors for SCID-X1 (REF.[34]). Lentiviral vectors have become the most commonly used vector for ex vivo HSPC gene therapy because of their good safety profile; lentiviral vectors preferentially integrate into gene bodies over promoters, reducing the potential for aberrant transcriptional activation (FIG. 4b). Furthermore, in self-inactivating vectors, the viral promoter located in the lentiviral vector LTR is inactivated on integration into the genome, limiting transcriptional transactivation of cellular genes. Modifications in vector structure, including the deletion of promoter viral sequences in the LTRs and the splitting of viral protein genes across separate plasmids provided in *trans* during vector particle production, have since contributed to a reduction in genotoxic risk associated with transcriptional transactivation and aided the production of replication-defective vector particles[35]. Although lentiviral vector-mediated gene therapy has an excellent clinical safety record, there remains a theoretical long-term risk of genotoxicity. In addition, the risks associated with high-dose chemotherapy (such as acute toxicity, infections, secondary tumours) — which is required in some cases

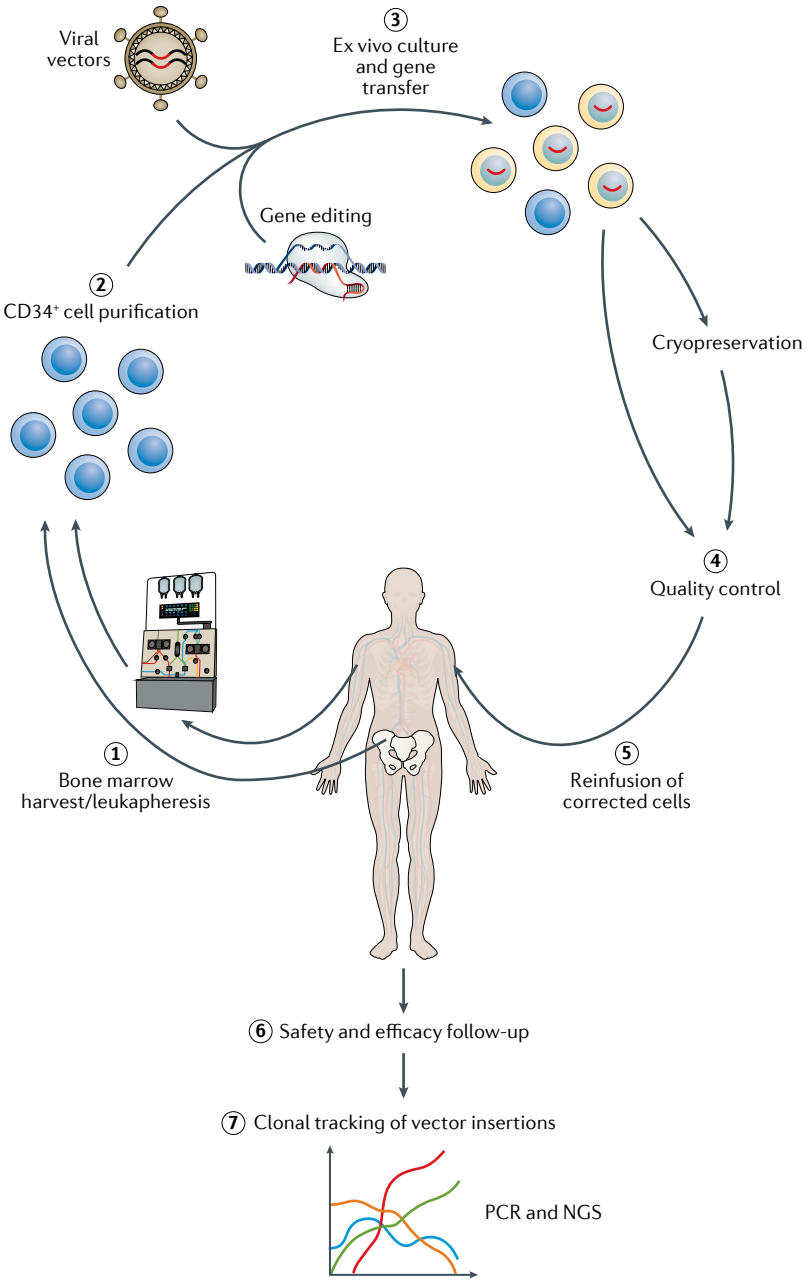

Fig. 3 | **Manufacturing of engineered HSPCs by gene addition and gene editing.** Autologous haematopoietic stem and progenitor cells (HSPCs) are collected from the patient through multiple aspirations from the iliac crests or by leukapheresis following the administration of growth factor and a chemokine antagonist. The collected material is enriched for $CD34^+$ cells cultured in the presence of cytokines and genetically modified either by gene addition using gammaretroviral or lentiviral vectors or gene editing using zinc-finger, CRISPR–Cas9 or transcription activator-like effector nuclease programmable endonucleases. Before gene therapy, a conditioning preparatory regimen is usually administered to patients to deplete endogenous HSPCs. The intensity of conditioning ranges from reduced intensity to myeloablative, depending on the disease and the engraftment level required to reach the therapeutic threshold. The medicinal product is represented by the gene-corrected cells, ready for infusion at the end of the manipulation or after a cryopreservation and thawing step. Quality control tests performed on the drug product may include those on viability, sterility, endotoxin level, mycoplasma, immune phenotype, number of vector copies per genome, transduction efficiency, transgene expression, vector production impurities and whether the vector is replication competent. In the case of fresh product or rapidly progressive diseases, a two-step strategy is used to allow urgent treatment without completion of all tests. NGS, next-generation sequencing. Adapted from REF.[184], Springer Nature Limited.

for HSPC gene therapy — hamper the application of this approach for less severe conditions. There are also limitations that prevent the wider application of gene therapy approaches for genes that require physiological control of gene expression or gain-of-function mutations.

Gene editing approaches

Site-specific genome editing using programmable endonuclease platforms such as CRISPR–Cas9, transcription activator-like effector nucleases or zinc-finger nucleases[36] can inactivate harmful alleles, disable transcriptional repressor expression or their binding sites, precisely correct mutations or insert healthy gene copies into a genomic 'safe harbour'. Precise gene correction may address potential issues associated with the semirandom integration of viral vectors, although it should be noted that so far no insertional mutagenesis has been reported with lentiviral vector-mediated gene therapy. Furthermore, targeted gene editing gives the advantage of bringing the therapeutic gene under the control of endogenous regulatory elements, allowing a physiological level of expression together with the potential of correcting diseases regardless of the type of mutation[37,38]. Recent studies showed proof-of-concept results in haemoglobinopathies[39] and the immunodeficiencies SCID-X1 (REFS[40–42]) and Wiskott–Aldrich syndrome[43].

DNA double-strand breaks induced by gene editing nucleases can be repaired through homology-directed repair (HDR) if a donor homologous template is provided, or by error-prone non-homologous end joining (NHEJ). HDR can be exploited to correct disease-causing mutations, whereas NHEJ leads to gene inactivation, potentially correcting gain-of-function and dominant-negative mutations or inducing therapeutic knockout. Ex vivo gene editing of HSCs has been shown to result in efficient NHEJ[44]; however, HDR in long-term repopulating HSCs occurs at low levels[40]. HDR may not be as efficient in long-term HSCs as in HPCs because it mostly occurs in cell cycle phases (S/G2) incompatible with the HSC quiescent state and because of inefficient donor template uptake by HSCs, which could limit the clinical translation of gene editing in diseases requiring a high frequency of gene-modified HSPCs such as lysosomal storage diseases. Chemical modification of single-strand guide RNA and innovations in the delivery systems of nucleases such as preassembled CRISPR–Cas9 ribonucleoproteins and the use of donor templates based on adeno-associated virus type 6 (AAV6) have increased the frequency of edited HSCs with promising results for future successful development and clinical application[39,44–47]. More recently, strategies to force cell cycle progression and to upregulate the expression of cellular components of the HDR machinery led to the successful increase of gene correction in up to 50% of in vivo repopulating HSCs[46].

Proof of principle for the gene editing of HSPCs has been achieved in preclinical studies[39,45,48,49], leading to two ongoing industry-sponsored clinical trials using zinc-finger nuclease and CRISPR–Cas9 technologies knocking out *BCL11A* to address transfusion-dependent β-thalassaemia and sickle cell disease (SCD), respectively

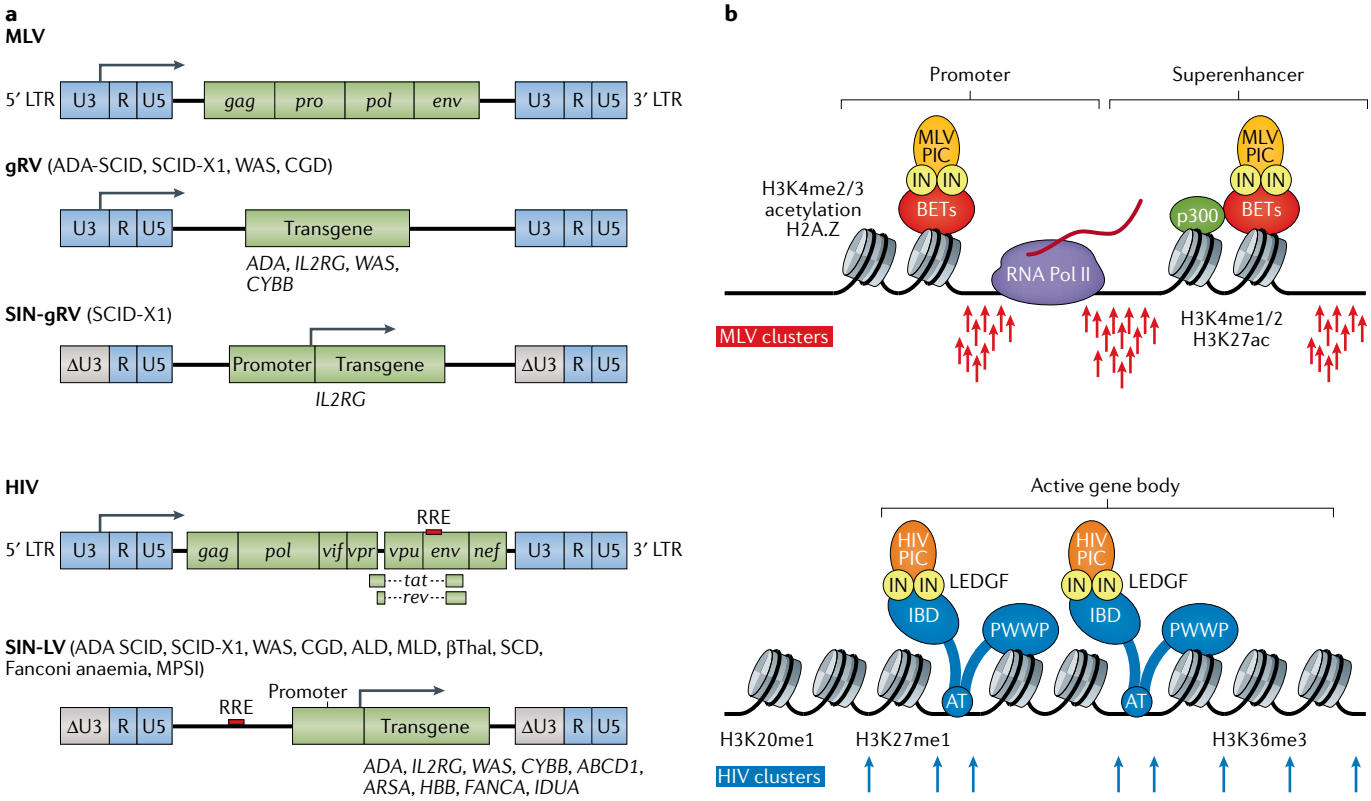

Fig. 4 | **HSPC gene therapy vector design and integration preferences.** **a** | Structure of the murine leukaemia virus (MLV) gammaretrovirus and HIV lentivirus genomes and derived vectors. Gammaretroviral (gRV) vectors harbour a viral promoter in the U3 region of the 5′ long terminal repeat (LTR). In self-inactivating gRV (SIN-gRV) and self-inactivating lentiviral (SIN-LV) vectors, deletions in the U3 region abolish the viral promoter, and the expression of the therapeutic transgene is instead driven by a promoter — generally of cellular origin — placed between the two LTRs. Diseases for which each vector is being investigated and the corresponding therapeutic genes are shown. Genes for viral proteins are provided in *trans* during vector particle production (not shown); the Rev response element (RRE) is included in the lentiviral vector backbone to increase viral titre. **b** | The differential distribution of MLV and HIV integration sites in the human genome is dictated by factors assembled in the pre-integration complex (PIC) tethering the vector DNA to specific chromatin regions. MLV-derived vectors preferentially integrate into promoters and enhancers, whereas lentiviral vectors integrate into active gene bodies. The bromodomain/extraterminal domain proteins are key proteins interacting with MLV PIC and influencing integration pattern of gRV and SIN-RV vectors. Lens epithelium-derived growth factor (LEDGF; also known as p75) mediates tethering of the lentiviral PIC to transcribed gene regions. Arrows indicate vector integration sites. βThal, β-thalassaemia; ADA-SCID, adenosine deaminase deficiency severe combined immunodeficiency; ALD, adrenoleukodystrophy; CGD, chronic granulomatous disease; H3K4me1/2, methylated/dimethylated histone H3 lysine 4; H3K27ac, acetylated histone H3 lysine 27; IBD, integrase-binding domain; IN, integrase; MLD, metachromatic leukodystrophy; MPSI, mucopolysaccharidosis type I; Pol II, polymerase II; SCD, sickle cell disease; SCID-X1, X-linked severe combined immunodeficiency; WAS, Wiskott–Aldrich syndrome. Part **b** adapted with permission from REF.[185], Elsevier.

(TABLE 1). *BCL11A* encodes a protein that represses the expression of fetal haemoglobin (HbF). Initial results from a patient with transfusion-dependent β-thalassaemia and a patient with SCD showed good editing efficiency of HSPCs using CRISPR–Cas9 to disrupt *BCL11A*, increasing levels of HbF and total haemoglobin over time in both patients, indicating successful engraftment of the edited cells[50]. SCD is an ideal model for HDR gene editing approaches as it is caused by a single-nucleotide mutation in a single gene. Studies using CRISPR–Cas9 to correct the SCD mutation — which is present in *HBB*, the gene encoding β-globin — showed HDR-mediated correction in isolated $CD34^+$ cells was effective in vivo, but the frequency of corrected cells decreased after xenotransplantation to less than 10%, indicating differential targeting of HPCs versus repopulating HSCs could limit the clinical application of this approach[45,51]. Further strategies for addressing SCD have exploited NHEJ to abolish the expression of BCL-11A. Disrupting either an erythroid-specific enhancer in the *BCL11A* gene[52] or BCL-11A-binding sites in the promoters of the γ-globin genes *HGB1* and *HBG2* (REFS[53,54]) led to an elevation in HbF levels in vitro. Immunodeficient mice into which $CD34^+$ cells corrected by CRISPR–Cas9 were injected showed a high frequency of edited HSCs and downregulation of *BCL11A* expression, and erythroid cells derived from edited engrafted cells showed correction of the SCD phenotype in vitro[44]. An alternative approach for targeting SCD has also been explored that mimics the condition of hereditary persistence of HbF through use of transcription activator-like effector nucleases and CRISPR–Cas9 to introduce deletions into the β-globin gene locus that interfere with gene regulation[55–58]. A proof-of-principle study using this strategy showed increased HbF expression and the correction of sickling in erythroid cells differentiated from patient $CD34^+$ cells, and the persistence of edited cells after transplantation in immunodeficient mice[58].

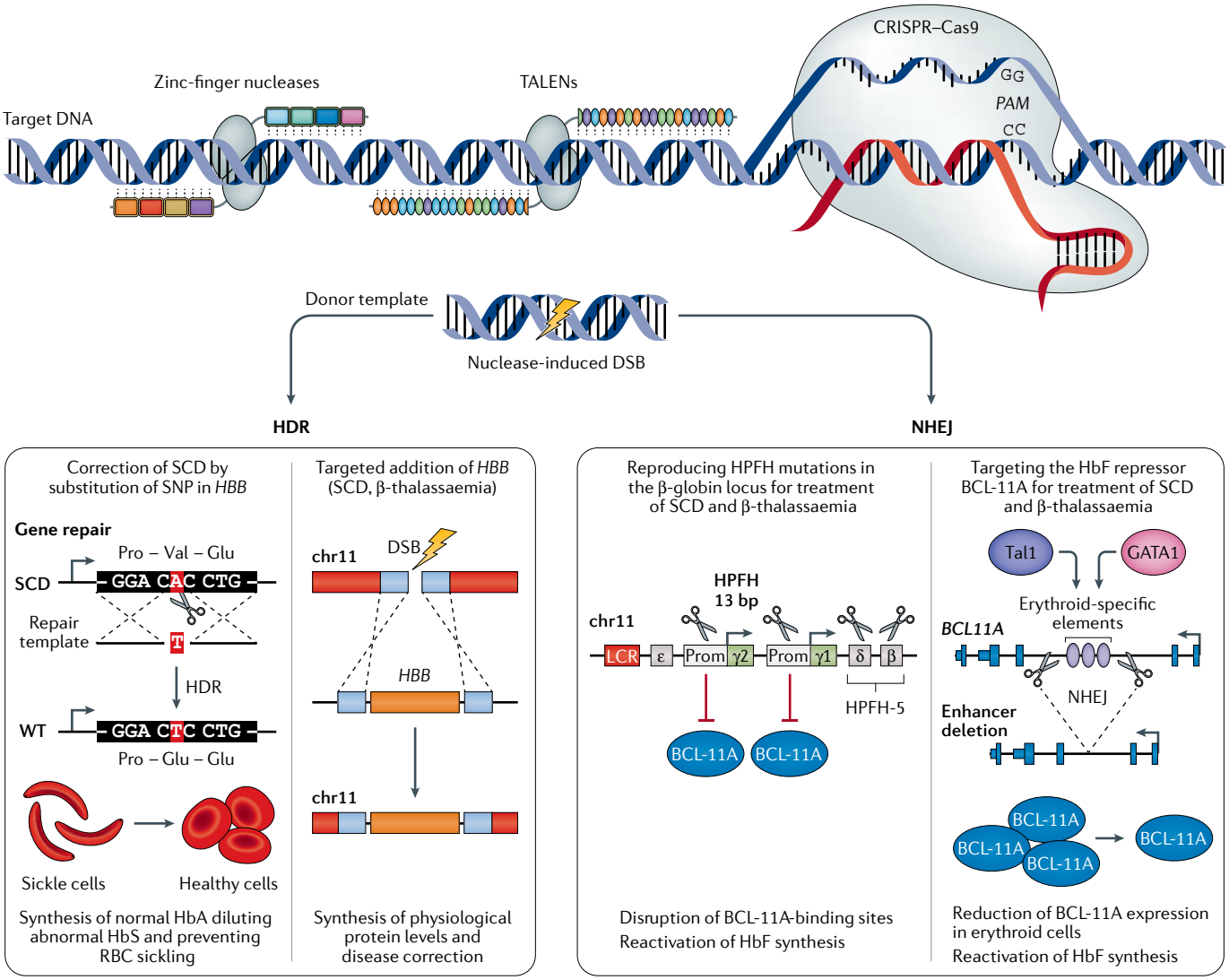

Fig. 5 | **Gene editing techniques for HSPC gene therapy.** Molecular tools for gene editing include zinc-finger nucleases, transcription activator-like effector nucleases (TALENs) and CRISPR–Cas9, which introduce targeted DNA double-strand breaks (DSBs) into the genome. The cell repairs the DNA breaks by non-homologous end joining (NHEJ), potentially introducing small insertions and deletions, or by homology-directed repair (HDR) if a suitable donor template is provided. Examples of gene editing approaches for the treatment of haemoglobinopathies are shown. HDR-driven strategies include correction of SNPs in the *HBB* gene locus to reverse red blood cell (RBC) sickling in sickle cell disease (SCD) and the targeted addition of *HBB* to treat either SCD or β-thalassaemia. NHEJ-driving strategies include the generation of mutations in the β-globin locus that mimic hereditary persistence of fetal haemoglobin (HPFH) and inhibition of the expression of *BCL11A*, the main repressor of fetal *HBG1* and *HBG2* genes, both resulting in the expression of fetal haemoglobin (HbF) and amelioration of the disease phenotype. chr, chromosome; HSPC, haematopoietic stem and progenitor cell; LCR, long coding region; Prom, promoter; WT, wild type. Elements of this figure are adapted with permission from REF.[186], Elsevier.

A summary of gene editing approaches for correcting haemoglobinopathies is given in FIG. 5.

Gene editing technologies do have potential risks associated with off-target effects and DNA rearrangements such as chromosomal translocations and large deletions[59], which are difficult to predict in preclinical models. In the case of HbF reactivation, the lack of a marker could make the origin of an increase in HbF level difficult to determine as increased HbF level is usually observed as a consequence of the transplantation procedure in general, especially in patients with SCD or β-thalassaemia[60,61]. Further advances in gene editing, such as base editing and prime editing[62–64] — which use a catalytically impaired CRISPR–Cas9 carrying nickase activity but lacking double-strand break activity in association with a deaminase and a reverse transcriptase, respectively — hold promise for efficient and possibly safer genome engineering, although most results have been obtained in cell lines and not in primary human cells.

Cell culture and transduction

In both vector-mediated gene transfer and gene editing approaches, the in vitro culture and manipulation of HSPCs can induce transcriptional responses and signalling events that may affect both primitive and gradually more committed cell populations[65,66] and reduce the frequency of gene-corrected long-term repopulating HSCs.

For this reason, a high dose of transplanted HSPCs is desirable both for fast haematopoietic recovery after conditioning and for a stable clinical outcome. Viral-mediated transduction of primitive HSCs requires prestimulation of the cells with activating cytokines such as stem cell factor (SCF), FMS-like tyrosine kinase 3 ligand (FLT3L) and thrombopoietin (THPO) and the use of high concentrations of a vector capable of high infectivity during ex vivo culture. The optimization of cell culture conditions such as culture duration and the choice or concentration of cytokines and transduction enhancers can enable the highest transduction efficiency or gene editing with the minimum loss of primitive HSC function. Indeed, culture time (usually between 24 and 48 hours) negatively correlates with the maintenance of repopulation capacity, as cytokine stimulation favours the expansion of more committed HPCs at the expense of repopulating HSCs[28,67]. Reliable assays to define transduction efficiency in long-term repopulating HSCs — one of the key factors for the prediction of a favourable outcome — are still lacking.

The potency specifications for a gene therapy drug product currently rely on surrogate parameters to measure potency. These parameters include the average number of vector copies per genome detected in HSPCs a few days after vector exposure, the proportion of successfully transduced clonogenic progenitors, the levels of transgene expression and corrected function. There are currently no available markers for the evaluation of repopulating HSCs following in vitro culture; consequently, multiple clinical trials have shown a lower rate of genetic modification in engrafted cells than in cells tested in vitro[24–26,68]. However, this is not the case for clinical trials investigating immunodeficiencies[13,14,22] and Fanconi anaemia[27], in which corrected cells are endowed with a proliferative advantage. The efficiency of transduction can be limited and variable among individuals, likely due to differences in the quiescence state of the isolated HSPC population and the expression of antiviral restriction factors by HSPCs[69] that act at different steps of the transduction pathway[7,70]. Recent advances using transduction enhancers ex vivo could increase transduction efficiency; for example, compounds such as poloxamers[71,72], rapamycin[73], cyclosporine A, cyclosporine H[70] and prostaglandin E_2 (REFS[28,74]) can enhance targeting of the vectors to HSPCs by enhancing vector particle attachment or entry, or by acting on postentry phases, allowing a higher level of integration. However, long-term clinical trials are needed to assess the efficacy of these drugs for increasing the efficiency of HSPC engraftment.

Patient conditioning

Conditioning regimens using chemotherapeutic or immunosuppressant drugs aim to deplete a patient's endogenous HSPC population in order to clear a niche for engraftment of corrected cells while mitigating toxicity. Conditioning can range from reduced intensity to myeloablative conditioning depending on the disease, the required level of transgene expression and the engraftment level required to reach the therapeutic threshold[75].

Myeloablative conditioning
High-dose chemotherapy that destroys haematopoietic cells in the bone marrow and severely reduces the number of blood cells. Usually followed by haematopoietic stem and progenitor cell transplantation or gene therapy to rebuild the bone marrow.

In autologous HSPC gene therapy, reduced-intensity conditioning allows the establishment of a stable mixed chimerism of uncorrected and corrected cells, which is possible due to the absence of rejection and graft-versus-host effects. This chimerism is favoured when corrected cells are endowed with an in vivo proliferative advantage; for example, in the case of lymphoid cells in immunodeficiencies[13,14,22] and corrected HSPCs in Fanconi anaemia[76]. However, reduced intensity conditioning strategies are usually insufficient for diseases such as lysosomal storage disorders or haemoglobinopathies[24,25,68,77] that require a high degree of engraftment. For example, myeloablative conditioning may be required for HSC gene therapy for β-thalassaemia in order to secure sufficient space in the bone marrow and allow the engraftment of an adequate dose of genetically engineered HSCs for differentiation into corrected red blood cells. For neurometabolic disorders, conditioning regimens based on alkylating agents are preferred due to their ability to cross the blood–brain barrier, deplete resident microglia and favour the local migration of corrected cells[78]. Toxicity associated with conditioning can be mitigated with use of pharmacokinetic techniques; for example, the toxicity of the alkylating agent busulfan is currently mitigated by serial evaluations of concentrations to determine the area under the curve[75], followed by dose adjustment.

Infertility is a major risk of myeloablative chemotherapy that can be addressed by gonadal cryopreservation in children[79]. Targeted and non-genotoxic methods for myeloablation as alternatives to conventional chemotherapy have recently been developed on the basis of antibodies that recognize HSC surface markers. Studies in mice and non-human primates demonstrated the efficacy of anti-CD117 (CD117 is also known as KIT) and anti-CD45 for the depletion of resident HSCs[80,81]. Recently, a phase I clinical study assessing the safety and tolerability of allogeneic transplantation in patients with SCID treated with an anti-CD117 showed promising initial results with successful engraftment of donor cells[82]. If proven to be safe and efficacious, these therapeutic antibodies could be used for conditions in which the risk of chemotherapy is deemed too high, or where the pre-existent inflammatory status of the bone marrow is further exacerbated by the effect of conventional conditioning regimens.

The bone marrow microenvironment plays a key role in facilitating the engraftment and expansion of genetically corrected cells. Recent studies found a defective bone marrow environment in β-thalassaemia was associated with impaired HSC function that could negatively affect engraftment[83,84]. A patient's age may also have an impact on bone marrow status and the quality of HSPCs; a recent study showed younger age was associated with better outcomes following HSPC gene therapy for transfusion-dependent β-thalassaemia[25].

Engraftment of modified HSPCs

Vector-transduced haematopoietic cells can be detected in the blood by quantitative PCR as early as 1 week after infusion. Corrected granulocytes and monocytes are detected first, followed by B cells, natural killer cells

and eventually T cells, which must first reach maturation in the thymus[13,85]. Most patients treated with lentiviral-mediated HSPC gene therapy display stable long-term engraftment of transduced cells and polyclonal haematopoietic reconstitution up to 8 years after treatment[8,22], and patients with ADA-SCID treated with gammaretroviral vectors show engraftment up to 15 years after treatment[86]. In both cases, engraftment results in persistent expression of the transgene driven by the integrated vector, as assessed by immunological and/or biochemical monitoring. Correction of the disease phenotype has been reported in most treated patients, the degree of which usually correlates with levels of engraftment and transgene expression in vivo. The extent of correction of repopulating HSPCs has been variable among trials, ranging from 0.1% to 80% of gene-modified HSPCs[7]. The highest and most consistent levels of engraftment for various lineages have been observed in studies using HSPCs corrected using lentiviral vectors and a myeloablative patient conditioning strategy[77]. Engraftment failures have been associated with insufficient doses of HSPCs or insufficient transduction efficiency, a lack of patient conditioning or the presence of concomitant diseases such as underlying infections[75,87–90].

The human bone marrow niche comprises several non-haematopoietic cells, including mesenchymal stromal cells (MSCs), osteoblasts, adipocytes, endothelial cells and neural cells, that offer physical support and secretion of soluble factors to HSPCs and regulate their homeostasis[91]. MSCs are localized in the endosteum and around the sinusoidal vessels[92] and can be classified into functionally distinct subpopulations identified on the basis of the expression of CD146 (also known as MCAM), CD271 (also known as NGFR), STRO-1 and stage-specific embryonic antigen 4 (REFS[93,94]). Humanized niche models are a powerful tool for dissecting the haematopoietic supportive function of distinct MSC subpopulations in vivo within a microenvironment that mimics human bone marrow[95,96]. MSCs have been used in the clinical setting to expand ex vivo HSPCs[97], and a recent pilot study described the infusion of MSCs to facilitate HSPC engraftment[97,98]. These studies suggest MSCs could be used in the context of HSPC gene therapy.

Vector integration and clonal tracking

Viral vectors retain the ability to integrate into the cell genome semirandomly, with some regions preferred to others depending on the class of retroviral vector used. Lentiviral vectors preferentially insert themselves into actively transcribed gene bodies, whereas gammaretroviral vectors preferentially integrate close to transcription start sites and active regulatory elements, specifically[99–104]. Semirandom vector integration could potentially induce changes in gene structure or expression that may provide a selective clonal growth advantage or result in uncontrolled proliferation[105]. The first vector integration studies in patients with ADA-SCID treated with gammaretroviral vectors showed the existence of insertion sites shared among multiple haematopoietic lineages, indicating bona fide engrafted, transduced HSPCs[102,106,107], although at the time these studies were conducted the efficiency of insertion site detection was low and most clones were derived from lymphoid lineages with a selective advantage[106,107]. Studies in immunodeficient patients showed that genes implicated in cancer are frequent insertion sites for gammaretroviral vectors; this may be as a result of preferential targeting for these integrations at the time of transduction[102] and/or because cells with these integrations are observed at a higher frequency due to clonal expansion of these cells after cell infusion[108,109]. Linear amplification-mediated PCR amplification and other methods that avoid restriction enzymes[105], coupled with next-generation sequencing of virus–host DNA junction sequences, have since been used routinely in clinical trials to monitor insertion site profiles and clonal fluctuation of the engrafted population[110,111].

Oligoclonality and enrichment of insertions near proto-oncogenes has been observed in patients treated with gammaretroviral vectors. Insertion site analyses have revealed the presence of integration sites near proto-oncogenes such as *LMO2* in leukaemic clones that developed in SCID-X1, Wiskott–Aldrich syndrome and CGD trials[15,108,112]. However, continuous insertion profiling was not able to detect the onset of leukaemia in patients who went on to develop the disease following HSPC gene therapy until clinical symptoms had become apparent[111]. Thus, the clinical application of insertion site analyses is currently limited to investigational studies and analyses of abnormal clinical findings, and not real-time monitoring.

A polyclonal pattern of vector integration has been observed in most patients at long follow-up times, with a balanced proportion of different clones. So far, no dominant clones enriched for vector insertion within oncogenes have been detected in lentiviral vector trials, with the exception of a single patient in a β-thalassaemia study[113]. The patient was found to have a dominant clone harbouring an integration in the *HMGA2* gene, causing deregulation of *HMGA2* expression that was not associated with adverse effects. The risk of genotoxicity for lentiviral insertions using a safe vector backbone and an adequate dose of HSCs is therefore predicted to be low[68,99,114]. Risk will further be reduced through safety monitoring of patients treated with lentivirus-modified HSPCs for at least 15 years[115].

Sequencing data generated as part of integration site analysis can be used to establish relationships between human haematopoietic lineages during distinct phases of haematopoietic reconstitution, as well as to estimate the number of long-term HSCs that participate in haematopoietic cell production after engraftment[116,117]. It has been estimated in patients treated with HSPC gene therapy that approximately $1/10^5$ to $1/10^6$ of the infused HSPC cell population contributes to long-term haematopoiesis[68,114]. Recent studies monitoring the kinetics of blood cell production from individual HSPCs suggest that distinct subtypes contribute to early and late post-transplantation phases differently. In particular, these studies showed that multipotent progenitors activate soon after transplantation and dominate initial haematopoietic output, whereas long-term repopulating

Oligoclonality
A quality associated with clones derived from one or a few cells or molecules.

HSPCs become predominant 1–2 years after transplantation, once haematopoiesis reaches a steady state[116,118]. However, it should be noted that these studies are based on data derived from gene therapy trials for PIDs and could be biased towards reconstitution of the lymphoid compartment. In cases where gene editing is used in HSPC gene therapy, studying the safety and dynamics of the corrected cell population could prove challenging owing to the lack of a distinctive element identifying each clone, especially when high-fidelity HDR is performed. Clonality studies may be useful in the case of NHEJ editing, since the DNA breaks produce insertions and deletions (indels) of different length that can be sequenced by next-generation sequencing techniques.

Applications for HSPC gene therapy

Primary immunodeficiencies

PIDs are a heterogeneous group of rare heritable disorders that result in an underdeveloped and/or functionally compromised immune system[119]. Patients with severe PIDs such as forms of SCID, combined immunodeficiency or severe myeloid cell disorders experience increase morbidity and mortality and display diverse clinical phenotypes[120]. HSCT using HSPCs from an HLA-matched donor can confer a lifelong 'cure', with a success rate of more than 90%[121–123]; however, the limited availability of HLA-matched donors, particularly in certain populations and non-Western regions, results in the necessity to use unmatched donors, which increases treatment-related risk due to graft-versus-host disease, toxicity and infections, the risks of which depend on age, co-morbidities and genotype[123]. PIDs less severe than SCID and combined immunodeficiency can still have a major impact on quality of life, and HSCT may be especially beneficial for conditions that require lifelong treatment with drugs and supportive therapies, which for many immunodeficiencies are only partially effective. Such treatments can also be limited by their availability and cost, particularly in the case of immunoglobulin replacement in antibody-deficiency syndromes or recombinant enzyme replacement therapies.

X-linked severe combined immunodeficiency. SCIDs are characterized by a depletion or functional deficiency of T lymphocytes and are often associated with B lymphocyte and/or natural killer cell deficiencies[120] As a result, these conditions are associated with high mortality in early life owing to severe infection. The success of HSCT gene therapy in treating many of these conditions is likely due to both a growth and survival advantage for gene-corrected cells and the inability of patients with SCID to reject allogeneic grafts. Because of these factors, successful engraftment of graft lymphocytes can occur without the need for cytoreductive conditioning[124]. The capacity for PID patients to accommodate donor lymphocytes, predominantly T cells, is unsurprising considering patients with PID can acquire somatic mosaicism and a milder or 'atypical' phenotype through spontaneous genetic reversions and second-site mutations[125].

SCID-X1 is caused by a deficiency of cytokine receptor common subunit-γ (also known as IL-2RG) — a critical component of multiple cytokine receptors, including the receptors for IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21 — which results in the absence of T cells and natural killer cells and the presence of functionally deficient B cells[126]. Early trials conducted using conventional gammaretroviral vectors with intact LTRs demonstrated rapid reconstitution of T cell immunity as a result of gene-corrected HSPC-mediated initiation of thymopoiesis in 17 of 20 patients treated[34,87] (FIG. 2). Humoral immunity was only partially restored, as the absence of cytoreductive conditioning and sustained HSC engraftment in these studies meant the resident B lymphocyte lineages remained uncorrected. Somewhat surprisingly, active thymopoiesis seemed to be maintained for many years after treatment[126,127], suggesting durable thymic engraftment of a very long-lived lymphoid or T cell progenitor even in the absence of corrected long-term repopulating HSPCs. However, these promising clinical data were tempered by the development of T cell acute lymphoblastic leukaemia in more than 25% of patients as a result of insertional mutagenesis[15,112]. The combination of semirandom vector genome integrations and potent enhancer sequences within the proviral LTR caused dysregulated expression of known proto-oncogenes, including *LMO2*, which has since been recognized as a major contributor to leukaemogenesis[128]. This, in combination with additional clonal genetic alterations — likely occurring stochastically or associated with proliferative stress — precipitated clinically manifesting leukaemia[15]. The occurrence of similar diseases in other trials not related to SCID-X1 (REF.[108]) using gammaretroviral vectors indicated the gene transfer technology was specifically inducing leukaemia. To address these issues, alternative vector platforms based on LTR enhancer-deleted gammaretroviruses[34] and more recently lentiviruses[129] have been used in a number of clinical trials for SCID-X1, with sustained immune reconstitution and clinical improvement and no evidence of clonal dysregulation or leukaemogenesis over more than 6 years of follow-up.

Recently, the use of low-intensity cytoreductive conditioning has facilitated successful long-term engraftment of gene-corrected HSCs and functional restoration of B cell lymphopoiesis as well as thymopoiesis in patients with SCID-X1 (REF.[130]). Older patients (adolescents and young adults) have also benefited from gene therapy with at least partial T cell recovery following failed allogeneic procedures, even though thymopoiesis has been dormant for many years[129]. This observation suggests that even at late stages, thymopoiesis is a retrievable developmental programme as long as there is a supply of gene-corrected T cell progenitors.

ADA deficiency SCID. ADA-SCID is an autosomal recessive metabolic disease caused by mutations in the gene encoding ADA which result in the accumulation of toxic metabolites such as deoxyadenosine that compromise lymphoid development. Enzyme replacement therapy through weekly administration of pegylated bovine or recombinant ADA can ameliorate the build-up of these metabolites in the blood and allows variable restoration of lymphopoiesis, albeit at a high financial cost[8]. Early attempts at gene therapy targeting residual mature T lymphocytes had minimal clinical benefit as patients

were maintained with enzyme replacement therapy[9,10]. However, the use of HSCs and the introduction of a low-intensity conditioning regimen to facilitate their engraftment and multilineage reconstitution produced remarkable clinical responses[14,88,131,132] (FIG. 2). Strimvelis, a gene therapy based on the introduction of ADA genes into HSCs using a gammaretroviral vector, was the first ex vivo gene therapy to be licensed, in 2016 (REF.[133]). Despite the presence of intact gammaretroviral LTR enhancer sequences and evidence suggesting clonal dysregulation occurs at the molecular level following treatment[134], no instances of clinically manifesting mutagenesis have been reported thus far[86,131,135]. [Note: after acceptance of this manuscript, an event of lymphoid T cell leukaemia has been reported in one patient, and its relationship to the gene therapy is currently under investigation (see Related links)]. Potentially, a leukaemic clone could be selected against due to its high requirements for the products of purine metabolism, which may be present at low levels in ADA-deficient patients[136]. Owing to the metabolic nature of the transgene and ADA being ubiquitously expressed, the ADA-defective bone marrow–thymus microenvironment could constrain haematopoietic or thymopoietic cells, protecting them from proliferative stress by partially compromising cell division and differentiation; however, this process is not fully understood. It should be noted that patients receiving enzyme replacement alone can also develop lymphoproliferation[137].

Other PIDs. Many other PIDs that are currently treatable using allogeneic HSCT are viable targets for an autologous gene therapy approach. Wiskott–Aldrich syndrome is a complex, multilineage PID caused by mutations in the *WAS* gene that result in haematopoietic cell cytoskeletal dysfunction, and is further complicated by thrombocytopenia[138]. Use of a conventional gammaretroviral vector to treat this condition resulted in clinical benefit but with an unacceptable degree of leukaemic toxicity[108,136]; however, a lentiviral platform incorporating a proximal segment of the *WAS* gene promoter has recently demonstrated sustained immunological correction and abrogation of bleeding tendency in more than 30 children and adults severely affected by Wiskott-Aldrich syndrome without clonal dysregulation[22,89,114,139]. Attempts at gammaretroviral correction of CGD — a group of conditions characterized by deficiencies of the NADPH oxidase system — were limited by lack of efficacy due to low engraftment, mutagenesis and transgene silencing as a result of LTR promoter methylation[109]. Haematopoietic proliferative stress and a decrease in the repopulating activity of HSCs have been observed in the CGD mouse model, suggesting that the chronic inflammation observed in patients with CGD could negatively affect the outcome of gene therapy unless it is adequately controlled[140].

Recent developments in the treatment of CGD have successfully restored long-term biochemical and immunological function in patients with severe CGD, allowing withdrawal of regular medications[90]. These developments include the refinement of a myeloablative conditioning regimen — necessary because myeloid cells, which are the predominant cell type affected by CGD, require continual lifetime renewal from HSCs — and the use of a lentiviral vector incorporating a regulatory element designed to avoid mutagenesis in HSPCs and mediate more physiological transgene expression patterns. This regulatory element is also being used in a clinical study for leukocyte adhesion deficiency type I, for which correction of the myeloid lineage is the principal goal. More recently, lentiviral vector platforms have been developed for the treatment of other forms of SCID, including those caused by deficiencies in *DCLRE1C*[141] (TABLE 1) and *RAG1* and *RAG2* (REFS[8,142]).

Erythrocyte disorders

The genetic diseases that affect red blood cells and are treatable by HSPC gene therapy include β-thalassaemia, SCD and pyruvate kinase deficiency. In SCD and β-thalassaemia, both of which are caused by mutations in *HBB*, gene therapy is particularly challenging because the inclusion of large-scale *HBB* genomic sequences and locus control region elements is a limiting factor in the design and manufacture of high-titre vectors[143]. From a safety point of view, the use of erythroid-specific regulatory sequences reduces the genotoxic risk of gene transactivation to erythroid precursors committed to enucleation and with a limited half-life.

β-Thalassaemia. In β-thalassaemia, mutations in *HBB* — the gene encoding the β-globin chain of haemoglobin — result in an imbalance between α-globin and β-globin chains that is toxic to erythroid precursors. β-Thalassaemia major is a particularly severe form of β-thalassaemia associated with chronic and severe anaemia caused by homozygous inactivating mutations in *HBB* (represented as β^0/β^0) and currently requires lifelong monthly blood transfusions and iron chelation therapy. Allogeneic HSCT has been used to cure β-thalassaemia, but is available only to the minority of patients who have a compatible donor[144].

HSPC gene therapy using erythroid-specific globin-expressing lentiviral vectors was the first strategy targeting β-thalassaemia successfully translated to clinical trials, in 2006 (TABLE 1). The safety and efficacy of the BB305 lentiviral vector, which encodes a β-globin transgene (β^{T87Q} globin) with antisickling properties, were reported in phase I and phase II trials of patients with differing severities of β-thalassaemia[24]. Clinical outcome was dependent on genotype, with 80% of patients with non-β^0/β^0 genotypes and 38% of patients with β^0/β^0 genotypes achieving transfusion independence at the 2-year follow-up. The remaining patients exhibited various levels of transfusion reduction. On the basis of these results, in 2019, the EMA gave conditional marketing authorization to Zynteglo for use in patients with transfusion-dependent β-thalassaemia with non-β^0/β^0 genotypes[145]. Optimization of the transduction protocol led to the start of two phase III clinical trials that are currently ongoing (TABLE 1, NCT02906202 and NCT03207009). In the TIGET-BTHAL clinical trial (TABLE 1, NCT02453477), nine patients with β^0/β^0 genotypes, including six minors, were treated with intrabone administration of GLOBE lentiviral vector-transduced

Iron chelation therapy
Pharmacological depletion of toxic iron accumulation in organs.

Stress erythropoiesis
The rapid development of new red blood cells stimulated in response to acute anaemia.

HSPCs. In the 1-year follow-up, the primary end points of transfusion reduction and safety were achieved in all patients, with four patients achieving transfusion independence. Patients in whom there was clinical benefit showed robust and stable engraftment of genetically modified cells in all lineages, including bone marrow erythroid cells[25]. Updated results showed a better outcome in minors than in adult patients[146]. Studies on the bone marrow microenvironment in β-thalassaemia and its potential impact on HSPC function[83,84] suggest the bone marrow microenvironment could impact clinical outcome; for example, impairment of stromal niche cells caused by disease-related secondary effects, such as ineffective erythropoiesis, iron overload or bone defects, has been reported in patients[83] and in thalassaemic mice with defective HSPC function in the latter caused by altered niche–HSPC crosstalk[84].

Sickle cell disease. The bone marrow microenvironment is a key factor for HSC quality in SCD, which is linked to a chronic inflammatory environment, an abnormal bone marrow vascular network and stress erythropoiesis[147,148]. SCD is caused by a single-nucleotide mutation in *HBB* resulting in the production of the toxic haemoglobin variant HbS, which polymerizes into long fibres that deform red blood cells into a sickle shape. Gene therapy strategies for SCD use transgenes encoding fetal γ-globin or β-globins with antisickling activity such as β^{T87Q} or βAS3, reactivate HbF production by reproducing mutations causing hereditary persistence of fetal haemoglobin (HPFH), or suppress the biological action of BCL-11A, a major repressor of γ-globin genes[149,150].

The first HSPC gene therapy strategy used to treat a patient with SCD used a BB305 lentiviral vector and bone marrow-derived HSPCs to deliver a transgene encoding antisickling β-globin. This approach achieved a therapeutic level of protein in erythrocytes corresponding to 50% of β-like globin chains, with successful correction of clinical symptoms[151]. Similar results were reported in one of two other treated patients. However, unsatisfactory results were obtained in seven initial patients treated in another trial[151]. Use of plerixafor-mobilized HSPCs instead of bone marrow-derived HSPCs and increase of the transduction level with a culture optimized protocol[152] has since improved the clinical outcome, with recent results showing sustained expression of transgenic antisickling β-globin and decreased SCD manifestations[153]. Two further phase I and phase II lentiviral vector-based clinical trials in SCD are currently ongoing; in one trial, the patients are subjected to reduced-intensity conditioning and transplantation with HSCs expressing the γ-globin gene[154], whereas in the other a lentiviral vector containing a β-globin transgene carrying three antisickling mutations (βAS3) was used. Early results have shown the treatment is safe and shows some benefit, although long-term follow-up will be needed to gauge efficacy. Finally, a different approach used lentiviral vector-mediated erythroid-restricted expression of a small hairpin RNA targeting *BCL11A* to reduce the repressive action of BCL-11A on HBG genes and consequently upregulate HbF levels[155]. A phase I clinical trial using this technology started in 2018 for patients with SCD (TABLE 1), with promising initial results showing HbF reactivation[156]. Long-term follow-up will be needed to confirm the origins of the increased HbF level and amelioration of clinical symptoms.

Pyruvate kinase deficiency. Pyruvate kinase deficiency is caused by a mutation in *PKLR* and is associated with haemolytic anaemia. Patients with severe pyruvate kinase deficiency often require blood transfusions and treatment with iron chelators. Lentivirus-mediated HSPC gene therapy transferring *PKLR* cDNA has shown proof of efficacy and safety in a mouse disease model[157], and a phase I/II clinical trial has recently been approved.

Bone marrow failure syndromes

Fanconi anaemia is a congenital, autosomal recessive DNA repair disorder associated with developmental abnormalities, bone marrow failure and a predisposition to cancers. A number of genes involved in DNA replication and repair have been implicated in Fanconi anaemia, including *FANCA* and *FANCB*. In a recent clinical study, patients with Fanconi anaemia caused by *FANCA* mutations received lentivirus-mediated HSPC gene therapy aiming to transfer an intact copy of the *FANCA* gene[27]. Patients were not conditioned so as to avoid drug toxicity. The study showed a gradual increase in corrected bone marrow $CD34^+$ cells, with the proportion of corrected cells ranging from 7% to 43% at 18–30 months of follow-up. Lentiviral vector-mediated gene therapy conferred a selective proliferative advantage to transduced HSCs and corrected blood cells by inducing resistance to DNA crosslinking agents as assessed in vitro. As expected, patients with the lowest marking levels in integration site analysis had the most limited number of repopulating clones fluctuating over time, and none of the treated patients has shown signs of genotoxicity so far. This approach was able to halt the progression of bone marrow failure in patients with the highest levels of gene-corrected cells. Recently, preclinical studies using CRISPR–Cas9-driven NHEJ to create compensatory mutations restoring the coding frame in HSPCs from patients with Fanconi anaemia showed a marked proliferative advantage for edited HPSCs in vitro and in mice in which xenotransplantation had been performed, with efficient correction of the Fanconi anaemia disease phenotype[158].

HSPCs as delivery vehicles

Inherited neurometabolic disorders

Mutations in genes encoding lysosomal or peroxisomal enzymes can cause the accumulation of toxic substrates in multiple organs, including the central nervous system (CNS); build-up of toxic substrates in the CNS specifically can lead to severe neurological damage[159]. Systemic administration of enzyme replacement therapy is approved for some metabolic disorders but its application for neurological disorders is hampered by the inability of proteins to cross the blood–brain barrier. Furthermore, skeletal deformities associated with lysosomal storage diseases remain difficult to treat with enzyme replacement therapy owing to the insufficient biodistribution of enzyme replacement therapy into

cartilage and bone[159]. These issues could be addressed with HSPC gene therapy as HSPCs and blood cells of the myeloid lineage are able to penetrate into the CNS and replace the resident populations of tissue macrophages and microglia-like cells[160,161]. Indeed, mouse models of transplantation using chemotherapy-mediated ablation of brain-resident myeloid cells have shown effective replacement of these cell populations with donor-derived HSPCs[78], and damage to microglia has been implicated in the pathogenesis of both peroxisomal and lysosomal storage diseases[162]. In the latter case, lysosomal enzymes secreted by corrected HSPC-derived myeloid cells that had migrated to the CNS could enable the correction of adjacent cell types — for example, oligodendrocytes, neurons and astrocytes — owing to the capacity of these cells to take up enzymes through the mannose 6-phosphate receptor present on their cell surface. Such enzymes include arylsulfatase A (ARSA) and α-L-iduronidase (IDUA), which are deficient in MLD and mucopolysaccharidosis type I (MPSI), respectively. HSPC gene therapy could also facilitate the replacement of functionally defective myeloid cells, including macrophages and microglia, restoring scavenging functions and contributing to amelioration of inflammation and oxidative stress in the brain[162,163] (FIG. 6).

Allogeneic HSCT is currently used as a therapeutic option in early forms of the peroxisomal disease X-ALD[164], and in selected lysosomal storage disorders, such as MPSI. However, some patients still experience significant neurological disease burden even after successful transplantation[165,166]. Allogeneic HSCT is currently ineffective in diseases such as mucopolysaccharidosis type IIIA (MPSIIIA) and in MLD especially in their early-onset forms[159]. This may be because of insufficient cross-correction of the metabolic defect in non-haematopoietic cells in the short time available to control rapid disease progression. Genetic modification of HSPCs designed to overexpress therapeutic proteins could therefore increase therapeutic protein levels in the blood and multiple tissues, including the CNS and bone, and provide enhanced cross-correction capacity, compensating for the presence of residual, uncorrected host cells. Individual neurometabolic disorders are discussed in the following subsections.

X-linked adrenoleukodystrophy. X-ALD is a severe demyelinating disease associated with a deficiency in ALD protein (encoded by *ABCD1*), which causes defects in the degradation of very long-chain fatty acids. The cerebral form of X-ALD is characterized by learning

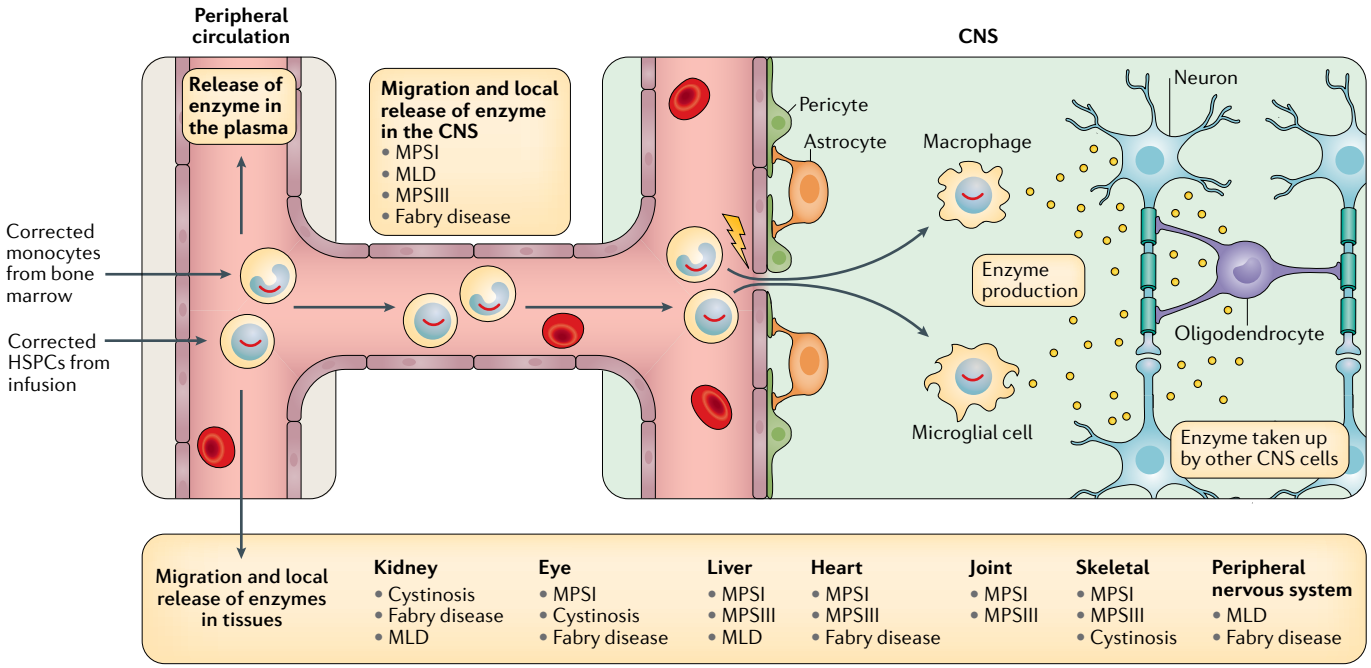

Fig. 6 | **HSPC-driven localized delivery of therapeutics in lysosomal storage diseases.** Lysosomal storage diseases are characterized by the accumulation of intracellular substrates caused by deficiencies in lysosomal enzymes. Substrate accumulation can disrupt normal cell function and can affect the function of organs such as the kidney, eye, liver, heart and bones, and the peripheral nervous system[77,165,170,171]. Gene-corrected cells may release functional enzyme into the circulation and at a local level following migration into the tissue to treat these diseases, as therapeutic enzymes can be taken up by non-corrected cells expressing mannose 6-phosphate receptor and break down accumulated intracellular substrates. Some lysosomal storage diseases affect cells of the central nervous system (CNS), leading to demyelination and cognitive and motor degeneration. To reach the CNS, cells need to cross the blood–brain barrier, which can be facilitated by chemotherapy (lightning bolt) and, in some cases, the underlying disorder. Haematopoietic stem and progenitor cells (HSPCs) may directly engraft in the CNS, expand locally and differentiate into corrected myeloid and microglia-like cells, which then release the therapeutic enzyme[78]. In addition, corrected myeloid progenitors or monocytes released from the bone marrow may migrate into the CNS, differentiate into macrophages and produce enzymes locally. The relative contribution of HSPCs and mature cells to correction is still unclear[165], although mouse models suggest a predominant role of progenitors[78]. HSPC gene therapy may be advantageous over enzyme replacement therapies for targeting the CNS due to the ability of HSPCs to cross the blood–brain barrier. MLD, metachromatic leukodystrophy; MPSI, mucopolysaccharidosis type I; MPSIII, mucopolysaccharidosis type III.

and behavioural problems starting at a median age of 7 years, followed by rapid neurological deterioration. Brain inflammation with infiltration of monocytes and microglia loss are hallmarks of the disease. The beneficial effects of transplantation are thought to be mediated by donor replacement of defective myeloid cells[26,162].

The first human gene therapy study with lentiviral vector-transduced HSPCs was performed in two patients with cerebral X-ALD[33] (FIG. 2). Gene therapy resulted in *ABCD1* transgene expression in 19% of $CD14^+$ myeloid cells, metabolic correction and clinical stabilization at 12–16 months after treatment. A subsequent trial saw similar results in 15 of 17 patients with X-ALD followed up at a median time of 29.4 months after gene therapy, who were free of major functional disabilities and had attenuated progression of brain lesions[26]. Peroxisomal enzymes are not secreted and the mechanism of disease amelioration for peroxisomal storage diseases is still not well understood, although it has been hypothesized that corrected cells can restore the metabolism of non-functional neuronal cells through direct intercellular contact[167].

Metachromatic leukodystrophy. MLD is a severe lysosomal storage disorder caused by mutations in *ARSA*, the gene encoding ARSA. ARSA deficiency results in the accumulation of sulfatides in the CNS and peripheral nervous system, leading to progressive demyelination and neurodegeneration. The first clinical trial based on HSPCs transduced with a lentiviral vector encoding *ARSA* showed safety and efficacy in eight of nine patients with MLD treated at a presymptomatic or very early symptomatic stage[68]. Treatment restored ARSA activity in patient circulating haematopoietic cells and in the cerebrospinal fluid to normal or above-normal levels — indicating local production by corrected HSPC-derived cells — and prevented either disease onset or disease progression. In contrast to untreated patients and their siblings, most treated patients displayed continuous motor and cognitive development[77] at a median follow up of 3 years; these results have since been confirmed in a larger cohort of patients up to 7.5 years of follow-up[168]. This gene therapy-based medicinal product (Libmeldy) has now received a positive opinion from the EMA Committee for Medicinal Products for Human Use (see Related links). This study represents a key proof of principle indicating that HSPC gene therapy could be used as a therapeutic strategy for lysosomal storage disorders.

Mucopolysaccharidosis. Mucopolysaccharidosis is a group of rare heterogenous diseases caused by deficiencies in enzymes involved in the breakdown of glycosaminoglycans. These diseases manifest themselves with somatic and neurological symptoms, depending on the type of accumulating glycosaminoglycan[169]. Individuals with the severe form of MPSI (Hurler syndrome), which is caused by a deficiency in the enzyme IDUA, usually develop skeletal abnormalities, hepatosplenomegaly, specific facial features, visual, heart and respiratory problems, and developmental delay, becoming severely intellectually disabled. The physical features of patients with MPSIII (Sanfilippo syndrome) are less pronounced than those of patients with MPSI, and children with MPSIII develop neurodegeneration with impaired cognition and behavioural and sleeping problems. Proof-of-concept studies in mouse models of MPSI and MPSIIIA — which is caused by mutations in the gene encoding *N*-sulfoglucosamine sulfohydrolase — treated with lentiviral vector-modified HSPC gene therapy have shown increased efficiency of this approach over allogeneic HSCT[170,171]. Clinical trials aimed at correcting the neurological phenotype and systemic features of MPSI and MPSIIIA have recently started (TABLE 1). A preliminary communication on the MPSI gene therapy study shows supraphysiological blood IDUA activity, rapid reduction of the levels of glycosaminoglycans and early signs of clinical improvement[172].

Other gene therapy strategies. Clinical trials based on lentivirus-transduced HSPCs are ongoing for the lysosomal diseases cystinosis and Fabry disease (TABLE 1) and are in the preclinical phase for MPSII, MPSIIIB and Pompe disease. It should be noted that the benefits of ex vivo HSPC gene therapy might be limited in symptomatic patients and patients with rapidly progressive disease variants because of the inherent delay in the enzymatic reconstitution of CNS tissues by HSPC-derived myeloid cells. Approaches based on direct intrabrain delivery of AAV or lentiviral vectors encoding the gene encoding the defective enzyme[173], intravenous injection of AAV vectors capable of targeting the brain, such as AAV9 (REF.[174]), or the direct injection of gene-modified HPCs[175] might provide more timely enzyme reconstitution. Universal newborn screening for early detection of these diseases is under development or already being implemented as part of established metabolic screening programmes and could allow prompt treatment before onset of symptoms[176].

Acquired diseases

HSPCs could be exploited to deliver therapeutic molecules systemically or to affected tissues for the treatment of acquired diseases such as cancers, acquired immune disease, chronic infections or neurodegenerative disorders such as multiple sclerosis. HSPCs could be engineered to produce cytokines that induce resistance to specific microorganisms or express surface receptors that modulate the immune response; for example, when expressed on HSPCs, the immune checkpoint molecule PDL1 was shown to inhibit autoimmune responses and reversed type 1 diabetes in an experimental model[177]. In addition, HSPCs could produce molecules blocking tumour cell growth or facilitating immune recognition of cancer. Local release of therapeutic molecules delivered by HSPCs could achieve sustained expression in target tissue while reducing systemic toxicity and the risk of adverse events. This was shown recently in preclinical models in which macrophage-infiltrating tumours derived from transduced HSPCs selectively expressing interferon-α induced an immunostimulatory programme in the tumour microenvironment, as shown by transcriptome analyses. This favoured T cell priming and effector functions towards multiple tumour antigens, leading to inhibition of leukaemia growth[178].

Conclusions and future perspectives

HSPC gene therapy offers the prospect of major clinical improvement and even cure for a large number of inherited immunohaematological and metabolic diseases. However, as autologous HSPC gene therapy reaches the clinic, there are significant financial and logistical challenges that must be addressed before its use on a global scale. Current strategies are based on centralized manufacturing facilities and a limited number of treatment centres that specialize in the disease and gene therapy, whereas fully automated transduction could modify this model by allowing local manufacturing and widespread diffusion of drug products.

The development of stable producer cell lines and reagents capable of enhancing viral attachment (such as LentiBOOST), suppressing natural intracellular viral restriction pathways (such as cyclosporine H) and promoting transduction while shortening ex vivo culture (such as prostaglandin E_2) are under intense investigation, with some already in clinical use[152,172]. Clonal tracking studies will be important to evaluate whether updated protocols preserve both primitive and committed haematopoietic progenitors in the drug product and favour rapid haematopoietic reconstitution, while simultaneously preventing rapid exhaustion of primitive HSCs and achieving long-term graft maintenance. Additionally, cryopreservation of the drug product or close automation of cell processing and transduction could allow greater standardization in terms of quality and dosing.

For most HSCT applications, the reliance on alkylating agents for patient conditioning is associated with short-term and long-term toxic effects. The development of antibody-based conditioning regimens targeting molecules expressed on host haematopoietic cells such as CD117 or CD45 could result in the replacement of alkylating agents or their use in conjunction with alkylating agents to allow a reduction in alkylating agent dose.

Recent preclinical studies have shown a potential alternative to ex vivo gene transfer through transducing HSPCs after direct intravenous in vivo administration of viral vectors, albeit at low efficiency[179].

In some cases, the physiological regulation of transgene expression is desirable to achieve a clinical effect and to avoid unwanted, transgene-related toxicity. Although incorporation of sophisticated regulatory elements into gene addition vectors may realize these criteria, locus-specific gene editing could harness natural gene regulatory mechanisms. However, the use of synthetic minigenes as 'universal' repair templates for homologous recombination will not necessarily recapitulate physiological gene expression and may require sophisticated design. Increases in gene editing efficiency, particularly in repair accuracy, will likely translate into an increasing number of clinical applications using gene-edited HSPCs. Although in principle gene editing should be safer than vector-based gene addition approaches as it should avoid issues with off-target DNA changes caused by semirandom vector integration, the clinical safety of gene editing in HSPC gene therapy has yet to be proven[180]. The development of high-fidelity Cas9 nucleases could overcome any residual off-target changes, although DNA double-strand breaks can also cause genome rearrangements such as deletions, inversions and translocations[59]. Base editing[134] and prime editing[62,63] hold promise for safer and more efficient genome engineering. Nevertheless, significant improvements in clinical-scale manufacturing and a better understanding of off-target and unexpected on-target effects are needed before implementation of gene editing in gene therapy. In the landscape of genome editing, preclinical studies and regulatory guidelines should be driven by previous experience from vector-based gene therapy, which has paved the way for the clinical translation of advanced cellular therapies. Assays and protocols must be adapted to better evaluate potential genotoxicity caused by gene editing.

Most HSPC gene therapies to date have targeted defined inherited diseases. As technology improves and our understanding of the durability and safety of HSPC gene therapy increases, opportunities may arise to target other disease settings. For example, acquired neurodegenerative conditions could benefit from sustained delivery of therapeutic molecules to the brain through HSPC-derived microglia. Chronic infectious diseases and cancer could also be ameliorated by systemic delivery of therapeutics, or through elimination of haematopoietic disease reservoirs as in the case of HIV[181]. The genetic modification of T lymphocytes with chimeric antigen receptor, for example, is an innovative approach to treat various forms of haematological cancer[182]. The use of HSPC gene therapy is likely to continue to grow rapidly and address an increasing range of immunohaematological and neurometabolic diseases.

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All authors researched the literature, provided substantial contributions to discussions of the content, and reviewed and/or edited the manuscript before submission.

Competing interests
The San Raffaele Telethon Institute for Gene Therapy is a joint venture between Fondazione Telethon and Ospedale San Raffaele. Gene therapies for adenosine deaminase-deficient severe combined immunodeficiency, Wiskott–Aldrich syndrome, metachromatic leukodystrophy, β-thalassaemia and mucopolysaccharidosis type I developed at the San Raffaele Telethon Institute for Gene Therapy were licensed to Orchard Therapeutics in 2018 and 2019. A.A. is the principal investigator in the above-mentioned clinical trials. A.J.T has equity in and is on the scientific advisory board for Orchard Therapeutics and receives consultancy payments from Rocket Pharmaceuticals.

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