Genetic Engineering and Manufacturing of Hematopoietic Stem Cells

Xiuyan Wang1,2,3 and Isabelle Rivière1,2,3

1Cell Therapy and Cell Engineering Facility, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA; 2Center for Cell Engineering, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA; 3Molecular Pharmacology Program, Memorial Sloan Kettering Cancer Center, New York, NY 10065, USA

The marketing approval of genetically engineered hematopoietic stem cells (HSCs) as the first-line therapy for the treatment of severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID) is a tribute to the substantial progress that has been made regarding HSC engineering in the past decade. Reproducible manufacturing of high-quality, clinical-grade, genetically engineered HSCs is the foundation for broadening the application of this technology. Herein, we review the current status of HSC transduction in the context of primary immunodeficiency diseases (PIDs) and other monogenic disorders.

Since the discovery of hematopoietic stem cells (HSCs) in the 1960s, HSC transplantation has become a curative clinical approach for an ever-increasing number of indications in oncology and regenerative medicine due to the unique self-renewal potential of HSCs.1 Given the experience with HSC transplantation, the genetic modification of autologous HSCs is an attractive therapeutic option for patients with monogenic disorders who lack a suitable HSC donor. By now, nearly two decades of clinical experience with the use of genetically modified autologous HSCs have been accumulated in patients with primary immunodeficiency diseases (PIDs). Although this treatment modality has proven to be efficacious for the treatment of PIDs such as severe combined immunodeficiency due to adenosine deaminase deficiency (ADA-SCID), X-linked severe combined immunodeficiency (X-SCID), and Wiskott-Aldrich syndrome (WAS), many challenges remain for the treatment of diseases such as chronic granulomatous disease (CGD) or hemoglobinopathies.2 Nonetheless, the recent approval of engineered HSCs for the treatment of patients with ADA-SCID provides an unprecedented proof of concept that paves the way for future applications.5 The genetic engineering and manufacturing processes are critical for the broadening of HSC-based therapies. Here, we review the current status of HSC transduction in the context of the largely successful clinical applications for the treatment of PIDs (X-SCID, ADA-SCID, WAS, and CGD) as well as other monogenic disorders such as adrenoleukodystrophy (ALD) and metachromatic leukodystrophy (MLD).

Manufacturing of Genetically Engineered HSCs

For patients with suitable donors, allogeneic HSC transplantation (HSCT) is accepted as the overt first line of therapy with proven efficacy in patients with immunodeficiency diseases such as ADA-SCID, X-SCID, and WAS. For patients without matched donors, clinical studies have demonstrated long-lasting and curative effects of genetically engineered autologous HSCs with the bona fide elimination of the risk of graft-versus-host disease (GvHD).2,4,7 The survival rate in patients with ADA-SCID has reached 100%, surpassing the efficacy of HSCT with fully matched donors.6 The successful manufacturing of genetically engineered HSCs is the basis for this unparalleled positive outcome. Processes ranging from HSC collection, CD34+ hematopoietic progenitor stem cells (HSPCs) selection, ex vivo activation/expansion, and genetic engineering of HSPCs must fulfill their own specific requirements to warrant a suitable clinical-grade product (Figure 1).

HPSC Collection

The primary sources for autologous hematopoietic progenitor and stem cells (HSPCs) are either bone marrow (BM) or mobilized peripheral blood (MPB). Several clinical trials have established that a minimum of $2 \times 10^6$ CD34+ cells/kg body weight is needed for successful engraftment, and $5-10 \times 10^6$ CD34+ cells/kg body weight is desirable for faster engraftment in the autologous setting.6,10 In their quiescent state, HSPCs are tethered to osteoblasts, stromal cells, and the extracellular matrix in the BM stem cell niche. BM has been collected and infused into patients for more than 60 years.11 When BM is chosen as the collection source, a surgical procedure is required. Patients undergo general or regional anesthesia and BM is collected through a needle inserted into the rear of the hip, which contains a large concentration of blood stem cells. When mobilized peripheral blood is chosen as the HSC source, HSCs need to be released from their BM niche to allow them to migrate into the bloodstream. The US Food and Drug Administration (FDA) has approved three agents for the mobilization of HSPCs: two hematopoietic growth factors, granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF); and a small bicyclam molecule, Plerixafor (also known as AMD3100). G-CSF is the first-line treatment for HPSC mobilization and has been shown to reduce neutropenia-related infection and enhance immunosuppression.
post-transplant myeloid recovery. GM-CSF is less efficacious in mobilizing HSPCs and considered a salvage mobilization regimen in patients who failed G-CSF mobilization. Plerixafor inhibits the CXCR4-SDF1 interaction within the BM microenvironment and induces rapid mobilization of stem cells. It has recently been shown that a combination of Plerixafor and G-CSF results in enhanced mobilization of HSPCs with greater repopulating potential in donors with multiple myeloma, non-Hodgkin lymphoma, and thalassemia, suggesting that distinct HPSC populations may be liberated from their specific niches by two different mechanisms. The COBE Spectra is the most commonly used platform for leukapheresis. The Spectra Optia system (Spectra Optia and the Spectra systems have been shown to generate comparable HPSC products, yielding the least amount of red cell contamination, whereas the Amicus system removes fewer platelets from the donor.

CD34+ HPSC Purification
CD34 has been used for the past 40 years as a surrogate marker for hematopoietic progenitors enriched with repopulating stem cells. Positive selection of CD34+ cells from BM or MPB is the method of choice for both autologous and allogeneic transplantations. Several immunoselection devices, including Ceparte, Isolex 300i, and CliniMACS have been used in the past for CD34+ cell selection. Currently, the Miltenyi CliniMACS Plus system together with the automated CliniMACS Prodigy are the only methods available for this procedure. CliniMACS offers the advantage of semiautomatic separation of magnetically labeled progenitor cells from both BM and MPB. It has been reported that a medium recovery of 71% and medium purity of 97% of CD34+ cells, with a median of 0.04% residual CD3+ cells could be achieved from either BM or MPB by using the CliniMACS device. Similar results were obtained with the automated CliniMACS Prodigy, although CD34+ cell recovery and depletion of CD3+ cells may be lower than with the semi-automated CliniMACS Plus instrument.

Vectors for HPSC Genetic Engineering
Multiple gene delivery vector systems, including adeno- or lentivirus, have been developed to provide either non-integrating transient gene correction or integrating stable gene transfer. For post-mitotic tissues, non-integrating vectors such as AAV vectors have been used in applications such as cancer, hemophilia, and inherited retinal dystrophy. For stable gene transfer, retroviral vector and lentiviral vector are the vectors of choice because of their ability to integrate into the host genome. Owing to HSPCs’ pluripotency, permanent correction of the gene of interest in HSPCs through genetic engineering provides a potential cure for patients who lack a suitable HSCT donor. Advanced sequencing technology and analytical tools have led to better understanding of the vector insertion pattern, which has guided a series of vector design improvements resulting in safer and more effective gene transfer strategies.

Gamma-Retroviral Vectors
The Moloney murine leukemia (MLV)-based γ retroviral vector (γRV) with intact long terminal repeats (LTRs) was among the first vectors employed for HSC gene therapy. More than 40 ADA-SCID patients have been treated with this vector, yielding 100% survival and 75% disease-free survival without detectable insertional mutagenesis. Despite marked clinical benefits in 20 X-SCID patients treated with CD34+ HSPCs transduced with MLV γRV expressing the common cytokine receptor γ chain, 5 of them developed T cell acute lymphoblastic leukemia due to γRV insertion-induced transactivation of LMO2 or CCND2 proto-oncogenes. Similarly, 7 of 10 patients treated with HSPCs transduced with MLV γRV encoding WAS protein developed leukemia associated with the dysregulation of LMO2 or with secondary myeloid malignancy as a result of insertional mutations in the MDS-Evi 1 locus. The γRV SF71pg91 vector in
| Disease (Gene) | Country | Study ID | HSC Source | CD34 Purification Method | Pre-stim Condition (Length) | Vector Type and Transduction Condition | Culture Length | Infusion Dose per kg (Medium) | Transduction Efficiency (No. Patients Treated) | Ref. |
|---------------|---------|----------|------------|--------------------------|-----------------------------|---------------------------------|---------------|-----------------------------|-----------------------------------------------|------|
| SCID-X1 (IL2RG) | France, UK, US | NCT01410019, NCT01175239, NCT01129544 | BM CliniMACS | 300 ng/mL SCF, 100 ng/mL MDGF, 60 ng/mL IL-3, and 300 ng/mL Flt3-L (24 hr) or 300 ng/mL Flt3-L (40 hr) | SIN γ-retro (EF-1α S) 3 rounds of transduction in 56 hr | 4 days | 3.7–10 × 10^6 (7.7 × 10^6) | VCN/cell: 0.25–2.92 (9) | | 47 |
| SCID-X1 (IL2RG) | US | NCT010028236 | MPB Isolex300i | 50 ng/mL SCF, 50 ng/mL Flt3 L, 50 ng/mL TPO, 25 ng/mL IL-6, and 5 ng/mL Flt3-L (16 hr) | γ-retro (MLV-LTR) addition of vector supernatant every day for 3 days | 5 days | 28.5–31.3 × 10^6 (29.2 × 10^6) | VCN/cell: 1.1, 2.3, 3.7 (3) | | 86 |
| SCID-X1 (IL2RG) | France | NCT 00599781 | BM CliniMACS | 300 ng/mL SCF, 100 ng/mL MDGF, 60 ng/mL IL-3, and 300 ng/mL Flt3-L (24 hr) or 300 ng/mL Flt3-L (40 hr) | MLV-LTR(GIADA) preloading of GIADA retroviral vector sup 33°C 4 hr, then incubate with CD34+ cells, 3 rounds of gene transfer in 3 days | 4 days | 0.9–13.6 × 10^6 (9.1 × 10^6) | VCN/cell: 0.35–2.2 (18) | | 87 |
| ADA-SCID | Italy | NCT 00599781 | BM CliniMACS | 300 ng/mL SCF, 300 ng/mL Flt3-L, 100 ng/mL TPO, and 60 ng/mL IL-3 (1 day) | MLV-LTR(SFada/W) 3 rounds of transduction in 56 hr | 4 days | <0.5–1.8 × 10^6 (3.2 × 10^6) | 5%–50% (6) | | 57,88 |

(Continued on next page)
| Disease (Gene) | Country | Study ID | HSC Source | CD34 Purification Method | Pre-stim Condition (Length) | Vector Type and Transduction Condition | Culture Length | Infusion Dose per kg (Medium) | Transduction Efficiency (No. Patients Treated) | Ref. |
|---------------|---------|----------|------------|--------------------------|-----------------------------|----------------------------------------|----------------|-------------------------------|-----------------------------------------------|------|
| US            | BM      | NCT 00018018 | ISOLEX 300i | CD34 Isolation            | 50 ng/mL SCF, 300 ng/mL Flt-3L, and 50 ng/mL MGDF (40-48 hr) | MLV-MPSV LTR and MLV-MND-LTR 3 rounds of transduction every 24 hr | 5 days | 0.7-9.8 × 10^6 (1.9 × 10^6) | VCN/cell: 0.1-13 (10) | 38   |
| UK, US        | BM      | NCT 01380990 NCT 02022696 NCT 01852071 NCT00598481 | NA | NA | (24 hr) | SIN-lenti (EF-1a S) 18 hr | NA | 3.17 × 10^6 (NA) | VCN/cell: 0.25-6.3 (20) | 4,53 |
| Germany       | BM or MPB | DRKS00000330 | CliniMACS | 300 ng/mL SCF, 300 ng/mL Flt-3L, 100 ng/mL TPO, and 60 ng/mL IL-3 | γ-retro (LTR) | NA | NA | VCN/cell: 1.7-5.2 (10) | 45,44 |
| WAS (WASP)    | BM or MPB | NCT01437242 NCT01437346 NCT02333760 | CliniMACS | SCF (300 ng/mL), Flt-3L (300 ng/mL), TPO (100 ng/mL), and IL-3 (20 ng/mL) (24 hr) | SIN-lenti (WAS promoter) LV-w1.6 WASp 2 rounds of transduction twice with 18 hr each time | 3 days | 2-11 × 10^6 (6.8 × 10^6) | VCN/cell: 0.6-2.8 (7) | 54   |
| Italy         | BM or MPB | NCT01515462 | CliniMACS | SCF (300 ng/mL), FLt-3L, 100 ng/mL TPO, and 60 ng/mL IL-3 (24 hr) | SIN-lenti (WAS promoter) LV-w1.6 WASp 2 rounds of transduction MOI of 100 | 3 days | 8.91-14.1 × 10^6 (10.3 × 10^6) | VCN/cell: 1.4-2.8 (8) | 45,55 |
| US            | BM      | NCT01410825 | CliniMACS | SCF (300 ng/mL), FLt-3L, 100 ng/mL TPO, and 60 ng/mL IL-3 (24 hr) | SIN-lenti (WAS promoter) | 5 days | NA | NA (2) | 3     |
| US            | NA      | MPB       | ISOLEX 300i | CD34 Isolation            | γ-retro (MLV-LTR) 3 rounds of spin-inoculation every 24 hr in 3 days | 4 days | 0.1-4.7 × 10^6 (2.5 × 10^6) | VCN/cell: 0.05-0.18 (5) | 89   |
| Germany       | MPB     | NA        | CliniMACS | 300 ng/mL SCF, 300 ng/mL Flt-3L, 100 ng/mL TPO, and 60 ng/mL IL-3 (36 hr) | γ-retro (SFFV-LTR) 3 rounds transduction 24 hr apart by incubating on freshly coated/preloaded flasks | 5 days | 3.6-5.1 × 10^6 (4.4 × 10^6) | P1: 45% P2: 39.5% (2) | 46,40 |
| Switzerland   | MPB     | NCT00927134 | CliniMACS | 300 ng/mL SCF, 300 ng/mL Flt-3L, 100 ng/mL TPO, and 60 ng/mL IL-3 (36 hr) | γ-retro (SFFV-LTR) 3 rounds transduction 24 hr apart by incubating on freshly coated/preloaded flasks | 5 days | 6.0 × 10^6 (6.0 × 10^6) | 32.3% (1) | 90-92 |
| UK            | NA      | NA        | NA        | NA | γ-retro (MLV-LTR or SFFV-LTR) | NA | 0.2-10.0 × 10^6 (NA) | 5%-20% (4) | 90   |

(Continued on next page)
| Disease (Gene) | Country | Study ID | HSC Source | CD34 Purification Method | Pre-stim Condition (Length) | Vector Type and Transduction Condition | Culture Length | Infusion Dose per kg (Medium) | Transduction Efficiency (No. Patients Treated) | Ref. |
|---------------|---------|----------|------------|--------------------------|-----------------------------|----------------------------------------|----------------|-------------------------------|-----------------------------------------------|------|
| X-ALD (ABCD1) | France  | NA       | MPB        | CliniMACS                | 100 ng/mL SCF, 100 ng/mL MDGF, 100 ng/mL Flt3-L, 60 ng/mL IL-3, and 4 ng/mL PS (19 hr) | SIN-lenti vector was added at MOI = 25 for 17 hr | 2 days        | 4.6 × 10^6, 7.2 × 10^6 (5.9 × 10^6) | P1: 50% P2: 33% (2) | 57,62 |
| US           | NCT01896102 | NA       | NA         | NA                        | NA                          | SIN-Lenti (Lenti-D)                     | NA            | NA                            | NA                                           | NA   |
| MLD (ARSA)   | Italy   | NCT01560182 | BM         | CliniMACS                | NA                          | SIN-lenti (ARSA-LV) 2 rounds of transductions 16 hr each time with 10^8 TU/mL ARSA-LV | 3 days        | 4.2–18.2 × 10^6 (9.9 × 10^6) | VCN/cell: 1.7–4.9 (4) | 58,62 |
| China        | NCT02559830 | NA       | NA         | NA                        | NA                          | NA                                      | NA            | NA                            | NA                                           | NA   |

NA, not available; BM, bone marrow; MPB, mobilized peripheral blood; SCF, stem cell factor; MDGF, polyethylene glycol-megakaryocyte differentiation factor; Flt3-L, Fms-like tyrosine kinase 3 ligand; TPO, thrombopoietin; GM-CSF, granulocyte-macrophage colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; MLV, murine leukemia virus; IL-3, interleukin 3; PS, protamine sulfate; LTR, long terminal repeats; SFFV, spleen focus-forming virus; MPSV, myeloproliferative sarcoma virus; MND, modified version of MPSV LTR with deletion of a negative control region and alterations of the adjacent primer binding site.38
which the gp91^phox subunit of the NADPH oxidase is expressed under the control of the spleen focus-forming virus (SFFV)-derived LTR was used in clinical trials to treat patients with X-linked chronic granulomatous disease (X-CDG). Although two patients treated with this vector demonstrated initial clinical benefits, both of them developed pre-leukemic myelodysplastic syndrome (MDS) due to the dysregulation of MDS1/EVI1.46

Self-Inactivating-Retroviral Vectors. Self-inactivating (SIN)-γRVs have been generated to improve the safety profile of γRV, in which the U3 promoter/enhancer is deleted from the LTR and the transgene expression is driven by an internal promoter. Such SIN-γRVs have been successfully used in X-SCID clinical trials, where the common cytokine γ chain expression is driven by the elongation factor 1α (EF1α) short promoter.47,48 Among nine patients, eight experienced stable correction of their immune cell functions, without retroviral vector insertion mutagenesis-related genotoxicity.47

Lentiviral Vectors. By using a tumor-prone mouse model, it was shown that lentiviral vectors (LVs) have a lower genotoxicity profile as compared to γRVs.49 This feature was also demonstrated in a sensitive in vitro immortalization (IVIM) assay performed in hematopoietic cells.50 SIN-LVs have become the vectors of choice in the most recently initiated clinical trials using HSCs. None of the patients who have displayed clear clinical benefits among the X-SCID (n = 5),51 ADA-SCID (n = 20),58,52,53 WAS (n = 27),3,43,44,54-56 ALD (n = 2),57 and MLD (n = 9)58,59 patients have undergone adverse events related to LV insertional mutagenesis post-autologous HSC gene therapy except one patient with thalassemia. In this patient, a single myeloid progenitor clone emerged upon trans-activation at the HMGA2 locus and eventually regressed after 7 years upon presumed exhaustion of the mutated clone. Importantly, this clone never progressed to leukemia.60 In-depth molecular analysis of the reconstituted hematopoiesis in patients with various diseases and treated with different types of vectors has allowed the characterization of vector-insertion distribution.51 Unlike the early intact γRV LTRs, chimeric LV LTRs elicit much less frequent dominant clones and display different insertion site preferences.54,55,62

CD34+ Cell Activation, Transduction, Formulation, and Cryopreservation
Efficient gene delivery and stable transduction of CD34+ cells require their pre-activation to exit cell cycle arrest. Gamma-retroviral vectors can only transduce dividing cells. Lentiviral vectors can infect non-dividing cells, but reverse transcription is minimal in G0-arrested cells.63 Upon collection and selection, the large majority of CD34+ cells from BM and MPB are in G0/G1 phases of the cell cycle; in addition, the BM-derived CD34+ cells contain approximately 10% of cells in S and G2/M phases.64 Stem cell factor (SCF) has been shown to effectively activate HSPCs for transduction when using a retroviral vector.65 The most commonly used activation conditions combine SCF, Fms-like tyrosine kinase 3 ligand (Flt3-L), thrombopoietin (TPO), and interleukin 3 (IL-3) for both γRV- and LV-mediated gene transfer (Table 1).

After pre-stimulation ranging from 14 to 40 hr, CD34+ cells are exposed to one or multiple rounds of transduction using either γRV or LV in presence of the same pre-stimulation cytokine cocktail. RetroNectin-coated tissue culture bags are frequently used to facilitate the transduction. The time in culture ranges from 2 to 5 days, resulting in transduction efficiency encompassing a wide range (Table 1). In order to minimize HSC differentiation and to maintain their pluripotency, the time in culture is kept short. In addition, maintaining the cultures for less than 4 days following the transduction allows archiving the quality control (QC) samples for replication-competent lentivirus (RCL) while alleviating the need to perform this expensive test.66 The transduced cell populations are subsequently harvested, washed, formulated, and released for patient infusion either fresh or upon cryopreservation.

For the trials listed in Table 1, Cartier et al.57 reported the use of cryopreserved cell products with hematopoietic recovery occurring at day 13 to 15 post-infusion, whereas other patients were treated with cells post-formulation without cryopreservation. For cryopreserved stem cell products, post-thaw viability constitutes one of the release tests. It has been recently reported that the viability of cryopreserved peripheral blood stem cells does not always correlate with the functional colony formation activity and engraftment outcome.67 Cryopreservation has a direct impact on the engraftment potential of stem cells from BM, peripheral blood (PB), and cord blood sources. Fast freeze rate is deleterious to clonogenic recovery and could be a major factor for engraftment failure.68-70 Thus, caution must be taken in designing rate-controlled freezer programs when using cryopreserved stem cell products.

HSC Gene Therapy Quality Control
Because the production processes are patient specific, moving forward this personalized cellular therapy toward a standard therapy poses great challenges. The manufacturing procedure needs to be carried out by trained manufacturing personnel under good manufacturing practices (GMPs) following validated conditions. The quality of genetically engineered HSCs is subject to donor-to-donor variability and is also dependent on the manufacturing environment, the quality of ancillary raw materials/reagents, and a robust, controlled, and reproducible manufacturing process to ensure product consistency with minimal risk of contamination. We previously published how our group validated the collection, transduction, and formulation of CD34+ cells derived from thalassemic patients genetically modified with a LV encoding a normal β-globin gene, prior to initiating the first phase I clinical trial approved by the FDA in the United States.71 Product quality is built within every manufacturing step and established through process qualification procedures and robust validation studies. More recently, we extensively reviewed the qualification of manufacturing processes and ancillary components for manufacturing chimeric antigen receptor T cells, which also applies to the clinical manufacturing of genetically engineered HSCs.72,73 Herein, we will therefore focus on the release testing of HSPC products.
The product testing and release criteria are devised to address the fundamental properties of the product including safety, purity, identity, and potency. Safety requires the lack of harmful contaminants, such as microbial agents, endotoxin, and mycoplasma; purity is reflected by the percentage of CD34⁺ cells and the transduction efficiency in the final product; identity of the product is commonly defined by the level of transgene incorporation in the genome; and potency of the transduced HSCs is often determined through soft agar colony formation assay and level of transgene expression in progenitors. Table 2 summarizes examples of release assays for RV or LV vector-transduced HSCs. The release of the cell products for infusion is handled through the issuance of a certificate of analysis, which summarizes the specifications and characteristics of the products and the release tests that have been performed.

Future Perspectives
Since the initiation of the first gene therapy trial in ADA-SCID patients in 1990,22,23 we have embraced the approval of HSC gene therapy as the first line of therapy for this disease.6 Cumulative evidence shows that HSC gene therapy is an effective treatment for various immunodeficiencies, inherited blood disorders, and monogenic metabolic diseases. More than 150 patients who did not have a matched donor have been treated with γRV or LV vector-transduced HSCs worldwide, the majority of which have demonstrated some clinical benefit (Table 1).

However, many challenges remain to be overcome. Standardization and automation of the manufacturing process await to be further developed. A very promising semi-automated system was recently reported to successfully transduce and manufacture non-human primate autologous gene-modified CD34⁺ cell products that were capable of stable, polyclonal multilineage reconstitution.26 This process is likely to be adapted to the transduction of human HSPCs.

It is advisable to further develop cell manufacturing processes based on quality-by-design (QbD) principles.76 In order for HSC-based therapies to realize their transformative potential, QbD principles should allow the linkage of measurable molecular and cellular characteristics of the cell population to final product quality.77 A well-designed manufacturing platform should take into account the complexity of process scheduling, traceability, and time to release given the unique nature of this personalized medicine.

Vector insertional mutagenesis is still a major safety concern. The safety profiles for lentiviral vector-based trials are encouraging51,55,59 and follow-up of these patients is ongoing. The maturation of gene-editing technologies, such as zinc finger endonuclease,78 TALEN,79 and CRISPR/Cas,80 also offers new prospects and promises for in situ gene correction and may allow to take advantage of the endogenous regulatory machinery to drive the physiological level of gene expression.81

The approval of HSC gene therapy for ADA-SCID is likely to widen the interest from industry and biotechnology companies, which will help develop automation platforms to enable commercialization. Certain existing platforms used in CAR-T cell manufacturing might also be suitable for genetically engineered-HSC production.72 The field of HSC gene therapy is evolving at a fast pace. Successful clinical applications are poised to promote this exciting treatment modality to the forefront of standard of care in the near future.

ACKNOWLEDGMENTS
We thank Michel Sadelain for thoroughly reviewing the manuscript. This work was supported by NCI grants P30 CA08748, NYSTEM grant N14C-010, Doris Duke Charitable Foundation grant GC202631, and Starr Foundation grant 2014-023.

REFERENCES
1. Müller, A.M., Huppertz, S., and Henschler, R. (2016). Hematopoietic stem cells in regenerative medicine: astray or on the path? Transfus. Med. Hemother. 43, 247–254.
2. Booth, C., Gaspar, H.B., and Thrasher, A.J. (2016). Treating immunodeficiency through HSC gene therapy. Trends Mol. Med. 22, 317–327.
3. Kuo, C.Y., and Kohn, D.B. (2016). Gene therapy for the treatment of primary immune deficiencies. Curr. Allergy Asthma Rep. 16, 39.
4. Cicalese, M.P., and Aiuti, A. (2015). Clinical applications of gene therapy for primary immunodeficiencies. Hum. Gene Ther. 26, 210–219.
5. Mansilla-Soto, J., Riviere, I., Boulad, F., and Sadelain, M. (2016). Cell and gene therapy for the beta-thalassemias: advances and prospects. Hum. Gene Ther. 27, 295–304.
6. Yla-Herttuala, S. (2016). ADA-SCID gene therapy endorsed by European medicines agency for marketing authorization. Mol. Ther. 24, 1013–1014.

7. Cavazzana, M., Six, E., Lagresle-Peyrou, C., André-Schmutz, I., and Hacene-Bey-Abina, S. (2016). Gene therapy for X-linked severe combined immunodeficiency: where do we stand? Hum. Gene Ther. 27, 108–116.

8. Touzet, F., Hacene-Bey-Abina, S., Fischer, A., and Cavazzana, M. (2014). Gene therapy for inherited immunodeficiency. Expert Opin. Biol. Ther. 14, 789–798.

9. Weaver, C.H., Hazlett, B., Birch, R., Palmer, P., Allen, C., Schwartzberg, L., and West, W. (1995). An analysis of engraftment kinetics as a function of the CD34 content of peripheral blood progenitor cell collections in 692 patients after the administration of myeloablative chemotherapy. Blood 86, 3961–3969.

10. Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A., Carral, A., de la Rubia, J., Martín, G., Martínez, J., Sanz, G., Jarque, I., Sempere, A.
LMO2-associated clonal T cell proliferation in two patients after gene therapy for SCID-X1. Science 302, 415–419.

41. Rowe, S.I., Mansour, M.R., Schwarzwaler, K., Bartholomae, C., Hubank, M., Kempski, H., Brugmann, M.H., Pfe-Overzet, K., Chatters, S.I., de Ridder, D., et al. (2008). Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. J. Clin. Invest. 118, 3143–3150.

42. Hacine-Bey-Ahina, S., Garrigue, A., Wang, G.P., Soulier, J., Lim, A., Morillon, E., Clapier, E., Caccurri, L., Delabesse, E., Beldjord, K., et al. (2008). Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. J. Clin. Invest. 118, 3132–3142.

43. Boztug, K., Schmidt, M., Schwarzer, A., Banerjee, P.P., Diaz, I.A., Dewey, R.A., Bochtler, M., Nowrouzi, A., Ball, C.R., Glumm, H., et al. (2010). Stem cell gene therapy for the Wiskott–Aldrich syndrome. N. Engl. J. Med. 362, 1918–1927.

44. Braun, C.J., Boztug, K., Paruzynski, A., Wind, M., Schwarzer, A., Rothe, M., Modlich, U., Bochtler, M., Göhring, G., Steinemann, D., et al. (2014). Gene therapy for Wiskott–Aldrich syndrome—long-term efficacy and genotoxicity. Sci. Transl. Med. 6, 227ra33.

45. Williams, D.A., and Thrasher, A.J. (2014). Concise review: lessons learned from clinical trials of gene therapy in monogenic immunodeficiency diseases. Stem Cells Transl. Med. 3, 636–642.

46. Ott, M.G., Schmidt, M., Schwarzwaler, K., Stein, S., Siler, U., Koehl, U., Glumm, H., Kühlke, K., Schilz, A., Kunkel, H., et al. (2006). Correction of X-linked chronic granulomatous disease by gene therapy, augmented by insertional activation of MDS1-EVI1. Mol. Ther. 14, 1417–1424.

47. Hacine-Bey-Ahina, S., Pai, S.Y., Gaspar, H.B., Armand, M., Berry, C.C., Blanché, S., Bleesing, J., Blondeau, J., de Boer, H., Buckland, K.F., et al. (2015). A modified γ-retrovirus vector for X-linked severe combined immunodeficiency. N. Engl. J. Med. 371, 1407–1417.

48. Stein, S., Ott, M.G., Schulze-Strasser, S., Jauch, A., Burwinkel, B., Kinner, A., Schmidt, M., Krämer, A., Schwäble, J., Glumm, H., et al. (2010). Genomic instability and myelodysplasia with monosomy 7 consequent to EVI1 activation after gene therapy for chronic granulomatous disease. Nat. Med. 16, 198–204.

49. Montini, E., Cesana, D., Schmidt, M., Sanvito, F., Ponzoni, M., Bartholomae, C., Sergi Sergi, L., Benedicenti, F., Ambrosi, A., Di Serio, C., et al. (2006). Hematopoietic stem cell gene transfer in a tumor-prone mouse model uncovers low genotoxicity of lentiviral vector integration. Nat. Biotechnol. 24, 687–694.

50. Modlich, U., Navarro, S., Zychlinski, D., Maetzig, T., Kutscher, I., Vidalin, M., Abel, U., Dal-Cortivo, L., Caccurri, L., et al. (2009). Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy: A phase I/II trial. Lancet 362, 818–823.

51. Castiello, M.C., Scaramuzza, S., Pala, F., Ferrua, F., Uva, P., Brigida, I., Sereni, L., van der Burg, M., Ottaviano, G., Albert, M.H., et al. (2015). B-cell reconstitution after lentiviral vector-mediated gene therapy in patients with Wiskott-Aldrich syndrome. J. Allergy Clin. Immunol. 136, 692–702.e2.

52. Cartier, N., Hacine-Bey-Ahina, S., Bartholomae, C.C., Veres, G., Schmidt, M., Kutscher, I., Vidalin, M., Abel, U., Dal-Cortivo, L., Caccurri, L., et al. (2009). Hematopoietic stem cell gene therapy in early-onset metachromatic leukodystrophy: an ad-hoc analysis of a non-randomised, open-label, phase 1/2 trial. Lancet 388, 476–487.

53. Sessa, M., Larioli, L., Fumagalli, F., Acquati, S., Reddelli, D., Baldoli, C., Canale, S., Lopez, I.D., Morena, F., Calabria, A., et al. (2016). Lentiviral haemopoietic stem cell gene therapy benefits metachromatic leukodystrophy. Science 341, 1233518.

54. Cavazzana-Calvo, M., Payen, E., Negre, O., Wang, G., Hehir, K., Fesl, F., Dewey, R., Denaro, M., Brady, T., Westerman, K., et al. (2010). Transfusion independence and HMG2 activation after gene therapy of human β-thalassemia. Nature 467, 318–322.

55. Biasco, L., Baricordi, C., and Aiuti, A. (2012). Retroviral integrations in gene therapies. Mol. Ther. 20, 709–716.

56. Büfi, A., Montini, E., Larioli, L., Cesana, M., Fumagalli, F., Plati, T., Baldoli, C., Martino, S., Calabria, A., Canale, S., et al. (2013). Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. Science 341, 1233518.

57. Wang, X., and Rivière, I. (2016). Clinical manufacturing of CAR T cells: foundation of regulatory and clinical development. Mol. Ther. 24, 1601–1607.
74. Onodera, M., Ariga, T., Kawaiamura, N., Kobayashi, I., Ohtsu, M., Yamada, M., Tame, A., Furuta, H., Okano, M., Matsumoto, S., et al. (1998). Successful peripheral T-lymphocyte-directed gene transfer for a patient with severe combined immune deficiency caused by adenosine deaminase deficiency. Blood 91, 30–36.

75. Adair, J.E., Waters, T., Haworth, K.G., Kubek, S.P., Trobridge, G.D., Hocum, J.D., Heinfeld, S., and Kiern, H.P. (2016). Semi-automated closed system manufacturing of lentivirus gene-modified haematopoietic stem cells for gene therapy. Nat. Commun. 7, 13173.

76. Rathore, A.S., and Winkle, H. (2009). Quality by design for biopharmaceuticals. Nat. Biotechnol. 27, 26–34.

77. Lipsitz, Y.Y., Timmins, N.E., and Zandstra, P.W. (2016). Quality cell therapy manufacturing by design. Nat. Biotechnol. 34, 393–400.

78. Urnov, F.D., Rebar, E.J., Holmes, M.C., Zhang, H.S., and Gregory, P.D. (2010). Genome editing with engineered zinc finger nucleases. Nat. Rev. Genet. 11, 636–646.

79. Ott de Bruin, L.M., Volpi, S., and Musunuru, K. (2015). Novel genome-editing tools to model and correct primary immunodeficiencies. Front. Immunol. 6, 250.

80. Sander, J.D., and Joung, J.K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. Nat. Biotechnol. 32, 347–355.

81. Genovese, P., Schirolı, G., Escobar, G., Di Tomaso, T., Fizzotti, C., Calabria, A., Moi, D., Mazzieri, R., Bonini, C., Holmes, M.C., et al. (2014). Targeted genome editing in human repopulating haematopoietic stem cells. Nature 510, 235–240.

82. Cavazzana-Calvo, M., Hacein-Bey, S., de Saint Basile, G., Gross, F., Yvon, E., Nusbaum, P., Selz, F., Hue, C., Certain, S., Casanova, J.L., et al. (2000). Gene therapy of human severe combined immunodeficiency (SCID)-X1 disease. Science 288, 669–672.

83. Hacein-Bey-Abina, S., Hauer, J., Aim, A., Picard, C., Wang, G.P., Berry, C.C., Martinache, C., Rieux-Laucat, F., Latour, S., Belohradsky, B.H., et al. (2010). Efficacy of gene therapy for X-linked severe combined immunodeficiency (SCID). N. Engl. J. Med. 363, 355–364.

84. Gaspar, H.B., Parsley, K.L., Howe, S., King, D., Gilmour, K.C., Sinclair, J., Brouns, G., Schmidt, M., Von Kalle, C., Barington, T., et al. (2004). Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. Lancet 364, 2181–2187.

85. Gaspar, H.B., Cooray, S., Gilmour, K.C., Parsley, K.L., Adams, S., Howe, S.I., Al Ghonaium, A., Rayford, J., Brown, L., Davies, E.G., et al. (2011). Long-term persistence of a polyclonal T cell repertoire after gene therapy for X-linked severe combined immunodeficiency. Sci. Transl. Med. 3, 97ra79.

86. Chinen, J., Davis, J., De Ravin, S.S., Hay, B.N., Hsu, A.P., Linton, G.F., Naumann, N., Nomicos, E.Y., Silvin, C., Ulrick, J., et al. (2007). Gene therapy improves immune function in preadolescents with X-linked severe combined immunodeficiency. Blood 110, 67–73.

87. Aiuti, A., Cattaneo, F., Galimberti, S., Benninghoff, U., Cassani, B., Callegaro, L., Scaramuzza, S., Andolfi, G., Mirolo, M., Brigida, L., et al. (2009). Gene therapy for immunodeficiency due to adenosine deaminase deficiency. N. Engl. J. Med. 360, 447–458.

88. Gaspar, H.B., Bjorkegren, E., Parsley, K., Gilmour, K.C., King, D., Sinclair, J., Zhang, F., Giannakopoulou, A., Adams, S., Fairbanks, I.D., et al. (2006). Successful reconstitution of immunity in ADA-SCID by stem cell gene therapy following cessation of PEG-ADA and use of mild preconditioning. Mol. Ther. 14, 505–513.

89. Malech, H.L., Maples, P.B., Whiting-Theobald, N., Linton, G.F., Seikahsa, S., Wovells, S.J., Li, F., Miller, J.A., DeCarlo, E., Holland, S.M., et al. (1997). Prolonged production of NADPH oxidase–corrected granulocytes after gene therapy of chronic granulomatous disease. Proc. Natl. Acad. Sci. USA 94, 12133–12138.

90. Grez, M., Reichenbach, J., Schwäble, J., Seger, R., Dinauer, M.C., and Thrasher, A.J. (2011). Gene therapy of chronic granulomatous disease: the graftenftime dilemma. Mol. Ther. 19, 28–35.

91. Bianchi, M., Hakkim, A., Brinkmann, V., Siler, U., Seger, R.A., Zychlinsky, A., and Reichenbach, J. (2009). Restoration of NET formation by gene therapy in CGD controls aspergillosis. Blood 114, 2619–2622.

92. Bianchi, M., Niemiej, M.J., Siler, U., Urban, C.F., and Reichenbach, J. (2011). Restoration of anti-Aspergillus defense by neutrophil extracellular traps in human chronic granulomatous disease after gene therapy is calpain-dependent. J. Allergy Clin. Immunol. 127, 1243–1252.e7.

93. Kang, E.M., Choi, U., Theobald, N., Linton, G.F., Kuhns, D., and Reichenbach, J. (2011). Restoration of anti-Aspergillus defense by neutrophil extracellular traps in human chronic granulomatous disease after gene therapy is calpain-dependent. J. Allergy Clin. Immunol. 127, 1243–1252.e7.

94. Kang, H.J., Bartholomae, C.C., Pizarzynski, A., Arents, A., Kim, S., Yu, S.S., Hong, Y., Joo, C.W., Yoon, N.K., Kuhns, D., et al. (2011). Retroviral gene therapy for X-linked chronic granulomatous disease: results from phase I/I trial. Mol. Ther. 19, 2092–2101.