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Hematopoietic Stem Cell Gene Therapy –Progress and Lessons Learned

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Abstract

The use of allogeneic hematopoietic stem cells (HSCs) to treat genetic blood cell diseases has become a clinical standard but is limited by availability of suitable matched donors and potential immunologic complications. Gene therapy using autologous HSCs should avoid these limitations and thus may be safer. Progressive improvements in techniques for genetic correction of HSCs, by either vector gene addition or gene editing, are facilitating successful treatments for an increasing number of diseases. We highlight the progress, successes, and remaining challenges toward development of HSC gene therapies and discuss lessons they provide for development of future clinical stem cell therapies.

Introduction

Most inherited blood cell diseases, such as primary immune deficiencies, hemoglobinopathies, storage and metabolic disorders, congenital cytopenias and stem cell defects, can be treated by transplantation of allogeneic hematopoietic stem cells (HSCs) (Table 1) (Boelens et al., 2013; Walters, 2015). The transplanted genetically normal HSCs can serve as an ongoing source of blood cells of all lineages, eliminating these disorders from a single treatment with benefits lasting life-long.

While there are generally high rates of success when an HLA-identical sibling donor is available, the outcomes of hematopoietic stem cell transplantation (HSCT) are usually not as successful with less well-matched allogeneic donors (either haplo-identical family members or unrelated donors) (Boelens et al., 2013; Walters, 2015). Reduced HLA matching between recipient and donor increases the risks of graft rejection and graft versus host disease (GVHD). Rejection of an HSC graft generally leaves the patient in a perilous position, with an urgent need to restore hematopoiesis to prevent complications from prolonged pancytopenia (anemia, infection, bleeding). The primary donor may not be available (e.g. cord blood units are not linked to their source) and a suitably matched second donor may not be identified. GVHD is a major cause of transplant morbidity and even mortality, and can impose a chronic rheumatologic-like inflammatory/fibrotic disease, with need for persistent

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immune suppression and the attendant risks of infection and toxicities (Cooke et al., 2017). Immediately before and after the allogeneic transplant, high levels of immune suppression are necessary to reduce immunological risks but these treatments also add to morbidity. There has been ongoing progress with methods to reduce GVHD in allogeneic HSCT, including improved graft engineering by removal of selective T cell populations (TCR α/β depletion, naïve T cell depletion), and by use of post-transplant cyclophosphamide (Fuchs, 2015; Muccio et al., 2016). Nonetheless, immune complications and lack of suitable matched donors present significant clinical barriers to successful application of allogeneic HSCT for a wider range of disorders.

Autologous HSCT in which the patient's HSCs are gene-modified should offer complete avoidance of the major immunological complications of allogeneic HSCT, which may contribute to better outcomes for patients with genetic blood cell disorders. For specific disorders, expression of the gene introduced into HSCs is needed in cells of one or more hematopoietic lineages (e.g. red blood cells, neutrophils, lymphocytes) (Figure 1). The lack of immunogenicity with autologous cells allows the use of reduced intensity of the pre-transplant conditioning to make space in the marrow niche to facilitate HSC engraftment, compared to what is required for effective allogeneic HSCT (Figure 2).

Current approaches to autologous transplant/gene therapy using lentiviral vectors (LVs) have produced clinical benefits similar to those from allogeneic transplant for several disorders. (Aiuti et al., 2013; Biffi et al., 2013; Cartier and Aubourg, 2010; De Ravin et al., 2016b). In multiple clinical trials (Table 1), this approach has consistently achieved quite stable frequencies of gene-corrected blood cells of all lineages, indicating engraftment, long-term persistence and ongoing generative capacity of gene-modified HSCs, with no significant diminution observed over time in human subjects (Cartier et al., 2012; Enssle et al., 2010).

Recent developments in gene editing have led to investigations toward its application for *ex vivo* gene correction in HSCs, which may have advantages compared to integrating viral vector-mediated gene addition (Carroll, 2016; Wright et al., 2016). This review will present the primary approach that is currently being used for gene modification of HSCs for clinical applications and gene addition using integrating viral vectors, as well as discuss the current status of gene editing in human HSCs for autologous transplantation. Lessons learned from advancing HSC therapies to the clinic may help inform the development of other stem cell therapies.

HSCs for Gene Therapy

HSCs are long-lived and multipotent, so gene correction in HSCs should lead to persistent gene correction among the different lineages (Kondo et al., 2003). The hematopoietic system is an ideal target for gene therapy because of the ease with which HSCs can be accessed for *ex vivo* gene manipulation, effective gene-modification, and re-administration as an intravenous infusion

HSCs are traditionally harvested from bone marrow derived from the iliac crests under general anesthesia. Multiple aspirations are performed with the goal of collecting 10–20 ml

of bone marrow per kilogram of recipient body weight. Alternatively, HSCs can be obtained as cytokine (e.g. G-CSF)-mobilized peripheral blood stem cells (PBSC) collected by leukopheresis. Hematopoietic growth factors, including GM-CSF and G-CSF, or CXCR4 inhibitors have been shown to increase the numbers of circulating hematopoietic stem and progenitor cells (HSPC) by 30–1000 fold (Brave et al., 2010). PBSCs are now the predominant clinical HSC source used for allogeneic and autologous transplants to routinely and successfully treat multiple blood cell disorders using current techniques.

However, the use of HSCs for gene therapy presents several challenges. HSCs are rare and delicate and are found among large numbers of more committed progenitors and mature blood cells that do not have long-term repopulating activity. While the immunophenotypic definition of unitary human HSCs has been well-developed, (e.g. CD34+, CD38−, CD45RA −, CD90+, CD49f+ (Notta et al., 2011), purification to high levels at clinical scale may entail significant losses of cells and impair their stem cell capacity. In current clinical practice for gene therapy, the HSCs from the clinical source (bone marrow or mobilized peripheral blood stem cells) are enriched, rather than purified, usually by isolating the CD34+ fraction using immunomagnetic separation. The CD34+ population (~1% of cells in adult bone marrow) contains most long-term engrafting multipotent HSCs, but also far more numerous short-term progenitor cells. CD34 selection enables ~30–50-fold enrichment of HSCs, removing the majority of highly numerous mature blood cells and enriching the HSC targets to culture for *ex vivo* gene modification. The dosages of CD34-selected cells typically used for transplantation range from 2 to 20 million/kg, necessitating efficient processing of relatively large numbers of cells.

Because they will divide many times, any gene modification of HSCs needs to be permanent and heritable to be passed on to all successive generations of progeny cells. Currently this necessitates making changes in the genome, either by covalent gene addition with an integrating vector or direct genome editing. The critical technical challenge for successful HSC gene therapy is performing sufficient gene engineering of the autologous HSCs to provide a therapeutic level of permanent genetic correction without impairing their stem cell capacity or causing adverse effects.

Thresholds for sufficiency can be based on observations from cases where patients, allo-transplanted for these disorders, develop mixed chimerism with only a sub-fraction of the hematopoiesis coming from donor cells. Clinical improvement has been reported with donor chimerism as low as 10–30% for sickle cell disease, thalassemia, SCID, and other PIDs, making this level a reasonable target for engrafted, gene-corrected HSCs (Chaudhury et al., 2017; Hsieh et al., 2011).

Vector choice and design

An attractive property of retroviruses is their ability to convert their RNA genome into proviral DNA through reverse transcription and integration into the DNA of the host cell’s genome in a quasi-random fashion. This integrating property of retroviruses allows the transmission of therapeutic information to all progeny of a transduced HSC. The initial retroviral systems used were derived from Murine Leukemia Viruses (MLV) a class of

simple gammaretroviruses (gRV) that were well-known from studies of their oncogenic properties. Transduction of human HSCs with gRV vectors has remained challenging due to the quiescent nature of HSCs that typically cycle infrequently during steady state hematopoiesis (Cheshier et al., 1999; Passegué et al., 2005; Pietras et al., 2011). MLV requires the breakdown of the nuclear envelope and cellular progression through mitosis to stably integrate into host cell genome as the virus lacks active nuclear localization elements (Lewis and Emerman, 1994; D. G. Miller et al., 1990). Therefore, HSCs need to be cultured for several days with multiple cytokines to induce cycling for retroviral transduction, and this may lead to loss of stem cell capacity.

Lentiviral vectors (LVs) have subsequently become the vector platform of choice because they do not require the cells to undergo mitosis for the breakdown of the nuclear membrane to efficiently integrate their proviral DNA into host cells. Rather, LVs transit through the nuclear pores by recruitment of host cell proteins. The most widely used design of LV system used for transduction of HSCs was first developed by Naldini *et al* in 1996 and was subsequently shown to efficiently transduce HSCs by Miyoshi *et al* and Case *et al*, among others (Case et al., 1999; Miyoshi et al., 1999; Naldini et al., 1996). While clinically-effective methods have been developed for gene introduction to human HSCs using retroviral and lentiviral vectors, HSCs are relatively resistant to transduction, requiring the use of high multiplicities of infection of vector (e.g. 10–100, based on titers measured on permissive cells) to effectively modify the majority of HSCs.

Safety issues

The first clinical trial utilizing gene therapy to modify autologous HSCs with curative intent began in 1992. The goal of this first trial conducted by Bordignon *et al* was to correct severe combined immune deficiency (SCID) syndrome caused by deficiency in adenosine deaminase (ADA) (Bordignon et al., 1995). SCID patients experience severe, recurrent and persistent infections resulting from immunodeficiency and, prior to the availability of HSCT options, the disease was lethal. This first clinical trial employing autologous gene-corrected HSCs to correct ADA-SCID utilized a vector derived from MLV to introduce an ADA cDNA into HSCs isolated from afflicted patients (Aiuti and Roncarolo, 2009; Gaspar et al., 2014). These investigators and other groups in the U.K. and the U.S. have gone on to treat more than 45 ADA SCID patients with gRV vectors, with good immune recovery in most and no complications from the vectors (Candotti et al., 2012; Gaspar et al., 2011; Shaw et al., 2017).

Additional trials using MLV-based gRV vectors, such as those conducted by groups in France and the U.K. for SCIDX1 (X-linked SCID) to correct interleukin 2 common gamma chain (IL2Rg) deficiency, demonstrated both the utility and the limitations of gRV vectors (Hacein-Bey-Abina et al., 2002). Although curative in the majority of patients, five (of 20 total) patients developed T cell acute lymphoblastic leukemia (T-ALL), two to six years post treatment, as a result of the action of vectors that had integrated near proto-oncogenes (Hacein-Bey-Abina et al., 2008; Howe et al., 2008). Similar leukoproliferative complications were seen in other clinical trials using gRV vector for X-linked Chronic Granulomatous Disease (X-CGD) (Ott et al., 2006), and Wiskott-Aldrich Syndrome (WAS) (Boztug et al.,

2010). The occurrence of T-ALL or myeloid malignancies in subsets of patients from each of these clinical trials was a result of the LTR driven gRV vector landing upstream of proto-oncogenes and ectopically activating their expression (Hacein-Bey-Abina et al., 2008; Howe et al., 2008). It is now known that gRVs tend to land near transcriptional start sites of genes, CpG islands, and DNAse 1 hypersensitive sites (which tend to be transcriptionally active) (De Rijck et al., 2013; Derse et al., 2007; Emery et al., 2009; Mitchell et al., 2004; X. Wu, 2003). The LTRs of these gRV act as strong enhancers that recruit a number of transcription factors capable of overriding innate cellular transcriptional control of neighboring genes, promoting leukemogenesis (Modlich et al., 2009).

To address MLV's propensity to induce enhancer-mediated insertional mutagenesis, Gilboa *et al* developed the first self-inactivating (SIN) vector by introducing a deletion within the 3′ U3 that abolishes enhancer activity. During reverse transcription, the deleted 3′ U3 is copied to both ends of provirus DNA and deprives the provirus of LTR mediated enhancer and promoter activities (Yu et al., 1986). Instead, internal promoters can be introduced to drive transgene expression with higher regulated/tissue specific expression. This alteration provides SIN vectors with increased safety by reducing cellular gene activation when in proximity to neighboring promoters (Nienhuis et al., 2006). SIN gRV vectors have been used safely for subsequent studies of gene therapy for SCIDX1, indicating that this modification did achieve its goal of greatly reducing risks of insertional transformation (Hacein-Bey-Abina et al., 2014).

These studies informed the development of LV as vehicles for gene delivery. Self-inactivating deletions were introduced into the viral LTRs of LV and all sequences encoding proteins supporting HIV virulence were deleted from the provirus and all packaging constructs to create second (-Vpr, -Vif, -Vpu, -Nef) (Zufferey et al., 1997) and third (also -Tat) generation LV vector systems (Dull et al., 1998). The VSV-G glycoprotein is most commonly used to pseudotype lentiviral vectors, although other envelope proteins have shown some favorable properties (Girard-Gagnepain et al., 2014). Lentiviral vectors can transduce non-dividing cells via several mechanisms they have for nuclear import of their viral cores (Matreyek and Engelman, 2013). They also have somewhat larger carrying capacity than gRV vectors (6–9 kb) and are generally more robust for transducing human cells. They have mostly become the vector of choice for stable gene addition to human HSCs.

Methods to optimize vector delivery

A major limitation of gRV vectors is their inability to transduce non-dividing cells efficiently. Addition to culture dishes of a specific adhesion domain of fibronectin in a recombinant protein, CH-206, which recruits virus particles to HSCs (resulting in a higher MOI at the interface between virus and cell) was found to significantly increase transduction efficiency (Hanenberg et al., 1996). *Ex-vivo* culture conditions were also found to influence HSC proliferation and transduction efficiency (Barrette et al., 2000; Sutton et al., 1999). Addition of recombinant human hematopoietic growth factors (typically ckit ligand, Flt-T ligand, thrombopoietin, and Interleukin-3) during transduction resulted in activation of CD34+ HSCs and therefore higher transduction rates. The combination of fibronectin and

optimal ex-vivo culture conditions greatly improved transduction with proven success in the clinic, as discussed below (Millington et al., 2009).

Another method that has been used to enhance transduction is to alter the cell target specificity of a viral vector by exchanging the innate envelope protein for one derived from an alternative virus allows researchers to alter the tropism of resultant vector particle (called pseudotyping). Typically, pseudotype is chosen based on expression level and exclusivity of the envelope protein's cognate receptor (higher receptor levels equal greater gene transfer levels). HSCs can be transduced with RD114, GALV, BaEV and VSV-G pseudotype viruses, among others. Other, more specific pseudotyping strategies are being developed that include the use of diverse viral envelopes and even fusions proteins with antibodies or cytokines to target specific cell types (Gennari et al., 2009; Verhoeyen et al., 2005).

Advances in vector design

Surprisingly, there have been no significant improvements to the basic design of lentiviral vectors since the so-called third generation vectors were introduced almost two decades ago (Dull et al., 1998; Zufferey et al., 1998). They were designed based on a decade of experience with gammaretroviral vector design and production and have met all the safety expectations, with no report of emergence of replication-competent lentivirus during packaging in research or clinical manufacturing.

Lentiviral vectors with relatively small and simple gene cassettes (e.g. human phosphoglycerate kinase gene or elongation alpha-1 gene minimal promoters and a cDNA) are readily produced to titers sufficiently high for effective gene modification of human HSCs at clinical-scale. However, low vector titer remains a significant problem with some LVs, especially those tasked to carry larger transgene cassettes, such as the human beta-globin gene. Several studies have shown that increasing viral RNA genome length negatively affects both titer and transduction efficiency (Cant Barrett et al., 2016; Kumar et al., 2004). Reduction of viral RNA length through removal of non-essential sequences is a viable strategy for improving LV titer for large transgene cassettes. Additionally, codon optimization may be used to improve titer by depleting secondary structures detrimental to mRNA stability (with the added benefit of improving transgene expression levels) (Moreno-Carranza et al., 2008). Other strategies for increasing LV titer include the addition of the Woodchuck Hepatitis Virus Post-transcriptional Regulatory Element (wPRE), which has been shown to increase vector titer through increasing vector genomic RNA stability, export and translation (Hope, 2002; Schambach et al., 2000) or addition of elements known to improve polyadenylation of vector mRNA during packaging, such as the Bovine Growth Hormone Polyadenylation Sequence (Woychik et al., 1984). Recently, Vink et al described a reconfiguration of the basic arrangement of the key cis-regulatory elements of HIV included in the vector backbone (LTRs, primer binding site, rev-responsive element) to simplify the process of reverse transcription, which may be a limit to transduction with large vectors (Vink et al., 2017). The advantages of this design in clinical applications remain to be tested.

To date, lentiviral vectors have been produced by transient transfection of multiple plasmids (vector, packaging proteins, envelope), which is cumbersome and challenging to scale-up to

levels that would be needed for commercial production. There have been extensive and long-standing efforts to develop stable lentiviral vector packaging lines, similar to those routinely produced for gammaretroviral vectors, which could provide a master cell bank for much simpler production of vector lots. It has proven to be challenging to repress expression of the VSV-G glycoprotein (which remains the most effective pseudotype for lentiviral vectors in most cases) and some of the HIV-1 genes which may be cytotoxic to the packaging cells for cell passaging, but then rapidly and robustly inducing expression of these genes for vector production. One stable packaging cell line has been used to produce lentiviral vector for clinical trials. (De Ravin et al., 2016b; Throm et al., 2009). It was made by successively transfecting plasmids encoding each gene cassette needed to make the virus proteins (HIV gag/pol, VSV-G) under tight, inducible expression control, and then concatamers of the vector plasmid to obtain multiple copies. This led to a cell line that was capable of scale-up to production volumes needed for production of clinical lots without loss of the packaging capacity.

Gene expression from lentiviral vectors

For some gene therapy applications, unregulated constitutive, ubiquitous expression of the transgene is acceptable. For example, adenosine deaminase (ADA) is expressed in all cell types and a broad range of ADA enzyme activity in all blood cell lineages is safe and sufficient to allow immune reconstitution. Thus, the vectors deployed for gene therapy of ADA SCID have used constitutive promoters, such as the MLV LTR or the Elongation Factor 1-Alpha gene promoter (Aiuti et al., 2009; Carbonaro et al., 2014). Other genes may require precise lineage, temporal or physiological-responsive expression patterns to be safe and effective. For example, molecules involved in signal transduction (receptors, intracellular signaling molecules, transcription factors) may be expressed in only specific cell types, or under specific physiologic states or in response to specific stimuli (e.g. *BTK*, CD40 ligand, *JAK3*, Stat proteins) (Brown et al., 1998). In some cases, it has been possible to build vectors using transcriptional control elements from endogenous cellular genes to apply regulated expression of transgenes (e.g. Beta-globin transcriptional control elements directing erythroid-specific expression of beta-globin (Sadelain et al., 2000). However, it may not be possible to incorporate into a vector the necessary regulatory sequences to recapitulate endogenous gene expression patterns for vectors integrated at an array of chromosomal sites in different cells. Insulator elements have been incorporated into some vectors to attempt to mitigate potential silencing of vector expression by heterochromatinization or trans-activation of adjacent cellular genes (Browning and Trobridge, 2016; Emery, 2011). The benefits of insulators remain theoretical and, in fact, silencing and trans-activation has been a problem with current lentiviral vectors. Endogenous gene expression is often controlled by enhancer and promoter interactions that occur over long distances (up to 100kb) away (West and Fraser, 2005). Regulated gene expression can be achieved to variable extents by adding a gene's known enhancer(s) upstream of a minimal promoter within the LV. These enhancers recruit and bind specific sets of transcription factors to cause *cis* activation of the promoter (Spitz and Furlong, 2012). The best studied example is the use of multiple elements from the β-globin gene locus to achieve erythroid-specific expression for the treatment of hemoglobinopathies (Cavazzana-

Calvo et al., 2010; Sadelain et al., 2000). However, it is sometimes difficult to fit all of the necessary cis-acting genetic elements required for precise gene expression within the size limit of vector genomes. In some cases, the function of enhancers can be antagonized by repressive chromatin structure at some LV integration sites (Hofmann et al., 2006; Yao et al., 2004). Chromatin domain insulators can be added into the LV's LTRs to overcome these positional effects and may also reduce risks from internal enhancers of vectors from affecting neighboring genes (Ramezani et al., 2008).

Lentiviral Vectors in Clinical Trials

Clinical trials using LVs began in the mid 2000's (Cartier et al., 2009; Cavazzana-Calvo et al., 2010) and LV have now been used safely and effectively in multiple studies for almost a dozen disorders using hematopoietic stem cells (Table 1) (and in many of the T cell-based immunotherapies with Chimeric Antigen Receptors and T Cell Receptor genes (Morgan and Boyerinas, 2016). In most trials, gene delivery to HSCs was at sufficiently high levels to produce clinically beneficial levels of gene-modified HSCs and relevant mature hematopoietic cells for the treatment of disease. The absence of GVHD and the reduced amounts of conditioning chemotherapy needed for engraftment of the autologous HSCs has allowed the predicted improved safety profiles. Analyses of LV integration site in the blood cells of subjects in the different clinical trials have shown a remarkably consistent pattern, with no predilection for insertion near proto-oncogenes and no clinically significant clonal expansions (Biasco et al., 2016; Biffi et al., 2011). LV gene therapy is being developed for several other disease indications (Table 1), including additional primary immune deficiencies, storage and metabolic diseases, and stem cell defects such as Fanconi's Anemia. Limitations to wider applications for more clinical indications are now less biological and more logistic, as each disorder requires its specific vector and the entire pre-clinical drug development pathway. Funding by research grants to perform vector development for additional indications may become more difficult to obtain, as the scientific novelty is diminishing; while funding from pharmaceutical companies and venture capitalists relies on the expectations of financial returns, which are unknown for these relatively rare orphan disorders. Issues related to commercial marketing and reimbursement for these cell and gene therapies are complex (Brennan and Wilson, 2014; Orkin and Reilly, 2016). Additionally, the capacity to produce the large volumes of clinical-grade LVs for these studies (and also for the larger studies of CAR T cells), is limited and may slow progress. Nonetheless, gene therapy using HSCs is continuing to advance and provide effective and safe therapies for a growing list of disorders.

X-linked Adrenoleukodystrophy (X-ALD) HSC Gene Therapy

A recent report described efficacious HSC gene therapy for X-linked Adrenoleukodystrophy (X-ALD), a progressive neurodegenerative disorder with onset in boys during the first 1–2 decades (Eichler et al., 2017). The major premise for the approach is that the gene-corrected engrafted HSC will produce cells that become CNS microglia and provide essential enzyme activity that can rescue very long chain fatty acid catabolism and prevent demyelination. Graft versus host disease, which seems to accelerate progression of X-ALD (Peters et al., 2004), is avoided with the use of autologous cells. The findings of beneficial stabilization of

neurologic status now extend and expand upon those of the initial report of two X-ALD patients treated using a lentiviral vector into CD34+ PBSC*[*1] (Cartier et al., 2009). The larger cohort studied by Eichler et al. (n=17) showed a high rate of response, with only one patient having progressive neurologic deterioration. HSC gene therapy effectively arrested disease progression in 88%, either without symptoms or with only initial progression to early neurological impairments followed by stabilization of neuro-imaging studies and clinical function.

This was the first commercially-sponsored clinical trial of HSC gene therapy (bluebird bio), with essentially all prior trials done at academic medical centers as research investigations. It was performed as a Phase II/II trial intended to obtain data to support applications for regulatory approval to market this stem cell gene therapy. Centralized manufacturing of the stem cell product was performed under full GMP conditions. Stem cell mobilization and leukopheresis was performed at several academic clinical sites enrolling subjects and shipped to a contract manufacturing organization for stem cell enrichment and transduction, yielding a cryopreserved product, which was shipped back to the clinical site following completion of product release testing. They produced consistently high quality cell products with good cell dosages (6–19x10^6 CD34+ cells/kg) and gene transduction levels (0.5–2.5 vector copies per cell). Patients were treated within 2–3 months from enrollment, which is an excellent time-frame to produce and certify a gene-modified stem cell product and perform all the clinical evaluations prior to a HSC transplant. The high rate of successful outcomes in terms of halting neurologic progression meet or exceed those of the clinical alternative of unrelated or haplo-identical donor transplants, strongly supporting the use of autologous gene therapy for this disorder (and by extension to many other storage diseases). Bluebird bio is pursuing regulatory drug approval for this combined cell and gene therapy product, which would represent one of the first approved gene therapies (bluebird bio, Inc., 2017).

Gene editing

Viral mediated gene transfer has well established benefits and has demonstrated clinical efficiency, as described above. However, risks and drawbacks of these methods remain, such as insertional oncogenesis and modified transgene expression pattern (Cesana et al., 2014; Ng et al., 2010; Zhou et al., 2016). Targeted gene editing allows for site-specific genome modification, and thereby eliminates the risks posed by randomly inserting genes. Another advantage of targeted gene editing over viral vectors is the ability to retain endogenous control of gene expression (Barzel et al., 2014; H. Li et al., 2011). Targeted gene editing can be achieved by employing site-specific endonucleases to induce a double-stranded break (**DSB**) in the DNA near the mutation site. The recruitment of DNA repair proteins to the site of DNA damage stimulates DNA DSB repair via one of two main pathways: non-homologous end joining (**NHEJ**), or homology-directed repair (**HDR**) (Figure 3). NHEJ can be used to achieve gene disruption with site-specific induction of a DSB by a targeted nuclease and NHEJ-mediated introduction of small insertions and deletions (**Indels**). NHEJ

*[1]Also the first clinical trial to use a lentiviral vector in human CD34+ cell transplants.

is a quick but error-prone pathway (Metzger and Iliakis, 2009); HDR is precise, but is dependent on cell cycle phase (S. Kim et al., 2014). Both of these endogenous DNA repair mechanisms can be harnessed for therapeutic benefit. In mammalian cells, NHEJ is more prevalent than HDR (Chiruvella et al., 2013). For a more detailed review on these DNA damage repair pathways, please see (Chapman et al., 2012; Heyer et al., 2010; Hustedt and Durocher, 2016; Lieber et al., 2003; Shrivastav et al., 2008).

Four main classes of endonucleases have been used for gene editing. The first, homing endonucleases recognize DNA sequences up to 40 bp long. These proteins are naturally found in six structural families (Jasin, 1996). The LAGLIDADG endonuclease family can be engineered to modify the sequence of DNA they recognize. The process is difficult and time consuming. An alternative being explored to facilitate retargeting HEs has been dubbed “megaTAL” (Boissel et al., 2013). These megaTALs feature a DNA binding domain composed of transcriptional activator like (TAL) effector DNA recognition motifs with the active endonuclease domain of a meganuclease.

Zinc finger nucleases (ZFNs), the second generation of engineered targeted endonucleases, provide a more easily modified system than homing endonucleases (Y. G. Kim et al., 1996). The enzyme functions as a dimer, with each ZFN containing three to five Zinc Finger protein motifs which recognize 3 base pair sequences of DNA and half of the *FokI* endonuclease complex. When a pair of ZFNs with appropriate target sequence binds closely enough for their *FokI* domains to dimerize, they make a DSB. ZFNs have promising function but are limited by the complexity of engineering new pairs.

Transcription activator like effector nucleases (TALENs) function similarly to ZFNs but use a different mechanism to recognize specific regions of DNA (Cermak et al., 2011). Instead of the zinc fingers, TALENs have 15–30 repeats of a 35 amino acid transcription activator like effector (TALE). Each TALE is composed of mostly invariable regions with only two amino acid differences known as repeat variable di-residues (RVDs). A TALE recognizes one base pair determined by which RVD a TALE contains. Adding a number of these TALEs together, fusing them to a *FokI* domain, and administering them in pairs, allows similar DSB formation to ZFNs but with an easier and more modular assembly.

The most recently described targeted endonuclease, Cas9, is a monomeric protein guided by a specific type of RNA, known as a CRISPR guide (Mali et al., 2013a). The guide RNA (gRNA) contains an 18–21-nucleotide long target sequence attached to a 3′ RNA scaffold loop for Cas9 protein binding. The target region must be complementary to a region in the DNA immediately upstream of a 2–5 base pair proto-spacer adjacent motif (PAM) which depends on the species of bacteria from which the Cas protein is derived. The most obvious advantage of CRISPR/Cas9 over the other nucleases is the ease and flexibility of developing guides to target new sites.

Gene Editing Strategies

Depending on the disease being targeted, the type of targeted editing required may fall into one of the three categories: gene disruption, gene correction, or gene insertion (Figure 3).

Gene Disruption

In certain cases, knocking out a regulatory element, viral receptor or a pathogenic gene may be sufficient to ameliorate the disease-causing phenotype. High levels of gene disruption may be achieved in hematopoietic stem and progenitor cells because this type of editing does not require a donor template and can be done via the NHEJ pathway. For example, disruption of the *BCL11A* erythroid enhancer (a repressor of fetal globin expression) can increase levels of fetal hemoglobin for the treatment of sickle cell disease and beta-thalassemia (Bauer et al., 2013; Bjurström et al., 2016; Canver et al., 2015; Chang et al., 2017). Alternatively, knockout of the *CCR5* gene in cells from HIV-infected individuals can prevent ongoing infection by the virus (Cradick et al., 2013; Hendel et al., 2015; Holt et al., 2010; L. Li et al., 2013; Mandal et al., 2014; J. C. Miller et al., 2010; Perez et al., 2008; Saydaminova et al., 2015; J. Wang et al., 2015). Trials targeting *BCL11A* are approaching the clinic (Chang et al., 2017) and several early phase clinical trials have been completed using ZFNs to modify the *CCR5* gene in HIV-infected patient peripheral blood T-cells (Tebas et al., 2014) or HSCs (DiGiusto et al., 2016).

Gene Correction

Diseases that result from a single nucleotide substitution or other small genetic lesions may be corrected by providing a homologous donor with the corrective sequence to serve as a template for DNA repair via the HDR pathway. Applications of this approach include correction of the sickle cell mutation in the beta-globin gene and restoration of beta-globin expression in beta-thalassemia. It is challenging to engage HDR-mediated repair in the quiescent, long term HSC population. In pre-clinical studies, gene correction levels of ~10%, ~25%, and ~35% using TALENs, ZFNs and CRISPR/Cas9, respectively, at the HBB locus were reported *in vitro* (DeWitt et al., 2016; Genovese et al., 2014; Hoban et al., 2015). However once the gene-edited cells were transplanted into immunocompromised NOD-scid-IL2Rg^{null} (NSG) mice, the gene correction levels decreased to below 10%. These data suggest that correction was less efficient in the true stem cell population than in more differentiated progenitor cells. Further studies must be performed to improve HDR in HSCs. Recently, a new mechanism of gene correction using a single-stranded donor template was reported, which is thought to act in a RAD-51 independent, and fanconi anemia (FA)-depended manner (Richardson et al., 2017). Modulation of FA pathway may be beneficial for improving the efficiency of gene correction.

Gene Insertion

In many human genetic diseases, there are a variety of different pathogenic mutations spread across the relevant gene in different patients. In general, the lengths of the gene repair tracts mediated by HDR are relatively short (<40 bp), so that it may be necessary to develop a panel of nuclease/donor template combinations capable of performing efficient editing to cover an entire gene region (Paquet et al., 2016). If this was accomplished using multiple CRISPR guides targeting different sites along a target gene or the genome, each guide might require validation for levels of activity and specificity, which could be impractical for Good Manufacturing Practice (GMP) production of the cells.

Instead, activity of the whole gene can be restored by the targeted insertion of a corrective cDNA of the relevant gene into the start of the endogenous gene locus. Examples of genes being targeted in HSCs by this method include: *IL2Rg* for X-linked Severe Combined Immune Deficiency (X-SCID), *BTK* for X-Linked Agammaglobulinemia (XLA), *CD40L* for X-Linked Hyper IgM Syndrome (XHIM), and *CYBB* for X-Linked Chronic Granulomatous Disease (XCGD) (Clough et al., 2016; De Ravin et al., 2016a; Genovese et al., 2014; Hubbard et al., 2016; Lombardo et al., 2007). Gene cassettes may be inserted into specific gene loci, such as *CCR5* for anti-HIV strategies to knock-out the viral co-receptor gene and simultaneously insert another anti-HIV gene; or into "safe harbors" such as the AAVS1 site which safely supports sustained transgene expression (De Ravin et al., 2016a; DeKelver et al., 2010; Hockemeyer et al., 2009; Lombardo et al., 2007; Mali et al., 2013b; J. Wang et al., 2015).

The donor template or cassette is flanked by homology arms surrounding the nuclease cut site and generally consists of the gene's full length cDNA complete with a stop codon and a 3′ untranslated region containing the polyadenylation signal. This cDNA donor template is most often delivered via an adeno-associated virus (AAV) or other non-integrating vector (J. Wang et al., 2015). A major challenge of this approach is to achieve efficient delivery and integration of these larger donor template. In pre-clinical studies, gene insertion rates of up to 43% at the IL2RG, AAVS1 or CYBB loci were achieved *in vitro*; however, as with gene correction, the levels of gene insertion in HSCs decreased *in vivo* (De Ravin et al., 2016a; Genovese et al., 2014; Schiroli et al., 2015; J. Wang et al., 2015).

Recently, two new methods of gene integration were described: homology-independent targeted integration (HITI) and precise integration into target chromosome (PITCh), which use NHEJ and MMEJ machinery, respectively (Nakade et al., 2014; Sakuma et al., 2015; Suzuki et al., 2016). The advantage of these strategies over HDR-mediated gene integration include being able to target cells outside the S/G2 phases of cell cycle.

Quiescence

HSCs are quiescent and mostly reside in the G0/G1 phase of cell cycle, providing a unique challenge for gene editing, since cell cycle phase is a major factor determining which DNA repair pathway is utilized to repair DSB. NHEJ occurs throughout the cell cycle while HDR is mostly restricted to S/G2 phases, when a sister chromatid is available to serve as a homologous template for repair (Branzei and Foiani, 2008; Pietras et al., 2011). This pattern of DNA repair has caused the rates of targeted gene correction and insertion in primary human HSCs to remain relatively low and rates of gene disruption by indels too high. Methods to increase HDR and decrease NHEJ are being developed. Current gene editing protocols utilize culture with a combination of recombinant hematopoietic growth factors (e.g. c*kit* ligand, FLT3 ligand, thrombopoietin and others) to induce cell cycling 24–72 hours prior to the delivery of nuclease and donor template. However, even with pre-stimulation the majority of HSPCs are not in S/G2 phases. Cell synchronization agents have been used successfully in 293T cells and the H9 embryonic cell line to temporarily arrest the cells in S/G2 phases of cell cycle during DNA repair to increase HDR (Lin et al., 2014). However,

the downstream effects of cell synchronization on the self-renewal and differentiation potential of HSCs is not yet known.

An alternative method being explored to improve the precision of gene insertion and gene correction is by simply reducing NHEJ. One such method reported to reduce NHEJ is the inhibition of Ligase IV, which is involved in the final step in the NHEJ pathway (Maruyama et al., 2015; Srivastava et al., 2012; Van Trung Chu et al., 2015). However, other groups have not been able to achieve a significant decrease in NHEJ using this inhibitor (Gutschner et al., 2016; Pinder et al., 2015; Yang et al., 2016). A potential concern with the inhibition of Ligase IV is that decreasing NHEJ levels in the cells may not result in an increase in HDR if the cells have already committed to the end-joining pathway. The effects of this type of late repair pathway blockade are still unknown, but it may lead to lower correction efficiency or even induce apoptosis.

Rather than preventing NHEJ via its terminal step, another possibility is to control the DNA repair pathway choice more upstream at the decision-making stage. For instance, formation of the BRCA1-PALB2-BRCA2 protein complex is crucial for HDR to occur; however, it is inhibited during G1 (Orthwein et al., 2015). Modulating the interaction between BRCA1-PALB2-BRCA2 in U2OS cells allowed Orthwein and colleagues to initiate HDR in the G1 phase of cell cycle (Orthwein et al., 2015). Whether this approach can be translated to primary human HSPCs remains to be tested. Another possibility is to decrease nuclease cutting in the G1 phase of the cell cycle by adding to Cas9 a fragment of the Geminin protein that causes Cas9 degradation during G1 when only the NHEJ repair pathway is available (Gutschner et al., 2016).

Methods of Delivery

One recurring challenge of targeted editing is how best to deliver the endonuclease and homologous donor template (if necessary for the particular treatment) to HSCs. Primary human HSCs are notoriously resistant to transfection methods of gene delivery (Van Tendeloo, 2001). Electroporation methods to deliver nucleic acids have improved over time and effectively transfer nucleic acid to the majority of HSCs in a treated sample. However, there is often a mild-moderate degree of toxicity from electroporation and this is significantly worsened by delivery of plasmid DNA, in some cases resulting in up to 60% cell death 24 hours post electroporation (Hendel et al., 2015). The delivery of *in vitro* transcribed mRNA encoding the nuclease and either *in vitro* transcribed or chemically-synthesized short guide RNA is better tolerated. Co-delivery of recombinant Cas9 protein complexed to short guide RNA as ribonucleoprotein (RNP) complexes has also been shown to be effective (S. Kim et al., 2014).

Delivery of homologous donor sequences has been achieved with multiple modalities. Chemically-synthesized oligonucleotides (e.g. 50–200 bp in length) are effective donors for small sequence changes, although they may cause moderate toxicity when introduced by electroporation (Hendel et al., 2015). Integrase-defective lentiviral vectors (IDLV), and the more effective adeno-associated virus (AAV) vectors can efficiently deliver donor sequences

of variable lengths (up to several kb) to HSCs with lower cytotoxicity than oligonucleotides or plasmids (Dever et al., 2016; Hoban et al., 2015; J. Wang et al., 2015).

Gene editing of HSCs for clinical applications

For clinical applications, multiple reagents are thus needed to perform gene editing (nuclease and donor) and each will need to be produced under standardized GMP conditions. Research scale editing is typically done with 0.2–1x10^6 CD34+ cells per experimental arm; clinical scale will involve at least 5–10x that many CD34+ cells *per kg*, and thus 50–1,000 times more cells. Although standards for acceptable levels of off-target cutting by a nuclease for clinical editing have not been defined, it is incumbent to investigate their occurrence with the most sensitive and relevant assays that can be practically done as part of pre-clinical toxicology assessments.

Challenges to Clinical Application of HSCs Gene Therapy

HSC harvest and expansion

While there has been much progress in applications of HSC gene therapy, many challenges remain. The numbers of HSCs that can be obtained from a patient are limited by the yields that can be isolated by bone marrow harvest or mobilization, although the combination of G-CSF and a CXCR4 inhibitor (plerixafor) generally leads to abundant cell collections (Brave et al., 2010). Some specific diseases may limit the numbers of HSCs that can be isolated, such as Fanconi anemia, which results in progressive HSC failure, or osteopetrosis, where the marrow space is progressively reduced by the accumulated bone (Daneshbod-Skibba et al., 1980; Giri et al., 2007).

The cell processing manipulations, including stem cell enrichment or gene modification--particularly when using electroporation--may lead to significant cell losses. Efforts to expand the numbers of true transplantable HSCs have been made, with several small molecules (such as SR-1, UM171, PGE_2) holding some promise, although no massive HSCs expansion has been achieved (Boitano et al., 2010; Fares et al., 2014; Goessling et al., 2011; Hoggatt et al., 2009; North et al., 2007). The goal of producing transplantable HSCs from pluripotent stem cells is advancing, with direct reprogramming to HSCs from endothelial cells also showing promise (Lis et al., 2017; Sugimura et al., 2017).

Gene Transfer

Gene transfer to HSCs has also advanced to a large degree, with current protocols of hematopoietic growth factor stimulation and transduction with lentiviral vectors reaching therapeutic efficacy for many disorders. Nonetheless, here too, improvements are needed. Human HSCs are relatively resistant to lentiviral vectors, evidenced by the seemingly high multiplicities of infection (M.O.I., the vector/cell ratio) needed to effectively transduce HSCs, compared to the relatively easier transduction of the cell lines typically used to gauge vector titers. The carrying capacity of lentiviral vectors has limitations, with vector titers falling off sharply as the size of the gene cassette increases. In our hands, a lentiviral vector at the small end of the size range (e.g. with a simple cDNA or transgene like GFP and a small promoter at ~4 kb proviral length) has a titer 10–30-fold higher than a vector at the

large end of the size range (e.g. with a beta-globin gene cassette with exons, introns, upstream locus control region segments at ~9kb). The lower production titer necessitates a proportionately higher volume of vector preparation to produce a patient dose, increasing the costs. Additionally, the bigger vectors do not transduce HSCs as well as smaller vectors, even when adjusted to matching MOI. Improved transduction of HSCs with lentiviral vectors using small molecules (proteasome inhibitors, cyclosporine A, rapamycin) has been reported in pre-clinical studies (Petrillo et al., 2015; Santoni de Sio, 2006; C. X. Wang et al., 2014), but the effects have not been clinically validated.And, of course, the semi-random integration of the vectors throughout the genome continue to pose genotoxicity risks, although these are greatly diminished with current generation vectors that lack the strong long terminal repeat enhancers that were the major cause of insertional oncogenesis with the first generation of gRV's.

Gene Editing

Gene editing may avoid many of the problems specific to viral vectors, but it too remains less than ideal in several ways. The various site-specific endonucleases (HE, ZFN, TALEN, or CRISPR) are fairly efficient and targeted gene disruption in HSCs is now in clinical trials for HIV (targeting CCR5, HIV co-receptor) and sickle cell disease (targeting BCL11a, repressor of fetal globin) (Chang et al., 2017; DiGiusto et al., 2016); clinical results have not yet been reported. However, the more elegant goals of targeted gene correction and gene insertion are more complicated to achieve, relying on the HDR pathway to perform the desired edits and thus requiring co-delivery of a homologous donor with the nuclease. Before clinical translation of gene editing, GMP methods of gene editing combining multiple GMP-grade reagents (e.g. CRISPR RNP or mRNA and short-guide RNA; AAV vector homologous donor) will need to be established. Pre-clinical work editing human HSCs using a clinically-scale approach has been reported (De Ravin et al., 2017).

Ex vivo processing

The *ex vivo* processing to enrich HSCs for clinical gene therapy has mostly been limited to CD34+ cell selection. This achieves a moderate (30–50-fold) decrease in total numbers of cells that need to be exposed to vector or gene edited (akin to a lineage-negative {lin−} murine population), but yet retains most of the HSCs. However, the CD34+ cell population is still quite heterogeneous with only a small fraction of cells being the target long-lived HSCs. Thus, a large proportion of the vector or gene editing reagents are wasted modifying the more abundant but short-term progenitor cells. Efforts to further enrich for HSCs using additional markers such as CD38(−), CD90(+), CD133(+), etc, (akin to a murine "LSK" {lin −/Sca1+/c*kit*+ fraction}) have been reported, but require FACS sorting which may entail long processing times and subject the cells to damaging shear forces (Baum et al., 1992). Newer sorting methodologies based on microfluidics or using serial immuno-affinity bead processes may be beneficial if they provide further enrichment without undue losses of cells (Masiuk et al., 2017; H.-W. Wu et al., 2010).

The cell culture methods used in current clinical trials are relatively standardized, using static culture in gas permeable bags or flasks in serum-free medium supplemented with multiple hematopoietic growth factors. The use of continuous feed bioreactors and/or lower

partial pressures of oxygen may provide more optimal conditions for HSC modification and preservation. Small molecules such as PGE_2 may also support HSC survival *ex vivo* improving the level of engraftment of gene-modified HSCs (Hoggatt et al., 2009). Additionally, the current *ex vivo* processing of HSCs is often done in multiple open systems, but new closed systems that continuously contain the cells are being developed that may allow processing to be done in environments less demanding than the current GMP "clean rooms".

Universal donor products

Universal donor cells could largely supplant use of autologous cell products if able to achieve the ideal properties of immunogenicity absence. They can be banked as an off-the-shelf, immediately-ready source of compatible normal cells, including regenerative stem cells. Universal donor cells would have a major advantage in that they could be produced in multi-patient dose lots vs. patient-specific single lots using autologous cell products. It is possible to engineer the cells to have favorable properties, e.g. produce a therapeutic protein such as clotting factors and other serum proteins, lysosomal enzymes, anti-tumor T cell receptor or Chimeric Antigen Receptor or an immunomodulative cytokine or chemokine. Allogeneic HSCT sources may continue to have advantages for HSCT for hematologic malignancies due to their potential graft-versus-leukemia effects, although it should become possible to augment specific immune effector cell products for the positive anti-leukemia effect, but without risks for GVHD.

Pre-transplant cytoreductive conditioning

Finally, the pre-transplant cytoreductive conditioning used to "make space" for engraftment of the isolated and reinfused HSCs is finally advancing beyond the use of cytotoxic chemotherapy drugs or radiation. These agents are effective at ablating the marrow stem cells (myeloablation), which is necessary for engraftment of gene-modified HSCs and for suppressing the recipient's immune system (immunoablation), which is necessary to avoid immunologic rejection of the graft. However, they may have severe acute toxicities in multiple organ systems (heart, lungs, liver, kidney, GI) and may produce infertility or sterility, due to toxicity to germ cells. Monoclonal antibodies to HSC surface proteins (e.g. ckit, CD47, CD45) have been shown in murine models to allow improved engraftment without apparent toxicity (Chhabra et al., 2016; Czechowicz et al., 2007; Palchaudhuri et al., 2016; Xue et al., 2010). These efforts are now being translated to the clinic and may eliminate the need to use toxic preparative regimens to facilitate engraftment.

Lessons learned

It takes a long time

Lessons learned from the almost 30-year history of developing clinical HSC gene therapy products can inform emerging stem cell-based cellular therapies for myriad other non-hematopoietic diseases such as Duchene's Muscular Dystrophy, Huntington's Disease, Parkinson's Disease, Diabetes Mellitus and others. One clear lesson has been that development of novel therapies takes a long time. Methods for effective gene transfer to HSCs were initially developed in the 1980s and clinical trials started in the 1990's. The

initial trials yielded no evidence of efficacy; the first clinical successes were not seen until the 2000's and only in the last decade are therapeutic benefits being conferred consistently for multiple disorders. Several promising HSC gene therapy cell products are advancing through early phase clinical trials (for indications including X-adrenoleukodystrophy, Metachromatic Leukodystrophy, Beta-thalassemia, Sickle Cell Disease, ADA-deficient and X-linked forms of SCID, Wiskott-Aldrich Syndrome, Chronic Granulomatous Disease) and towards licensure for commercial manufacture and sales, with the first (Strimvelis for ADA-deficient SCID) approved by the European Medicines Agency (European Medicines Agency, 2016). While it may be expected that therapies using other stem cell types will be derived in a shorter time-frame, drug development remains a slow process.

Support academic medical centers to develop novel cell therapies

HSC gene therapies were incubated at academic medical centers in multiple countries often at innovative HSCT programs, not via the traditional pharmaceutical company model of drug development. Universities and other research centers need to have sufficient infrastructure for early phase clinical trial performance and GMP cell processing to achieve similar academic pioneering for other stem cell therapies. Indeed, the centers that have had strong gene therapy programs with the necessary cell processing and regulatory infrastructure have been the leaders in this field (e.g. TIGET, Milan Italy; Hôpital NeckerEnfants Malade, Paris, France; University College London, London, UK; The National Institutes of Health, Bethesda MD; University of California, Los Angeles, Los Angeles CA; Boston Children's Hospital, Boston MA; St. Jude Children's Research Hospital, Memphis TN). Ongoing support of this type of research will be essential to continue the innovation of new therapies.

Do it right

In the early days for the field of gene therapy, the NIH RAC-provided public oversight to review clinical protocols to be performed in academic medical centers was an important forum to discuss the novel potential biohazard issues, as well as scientific and ethical concerns. This may have tempered or even slowed some advancements, but it helped provide another level of expertise, in addition to that provided by local IRB and FDA (and EMA in Europe), to ensure that trials were based on sound scientific principles, had adequate supporting pre-clinical data on potential efficacy and safety, and were well-designed and monitored. The role of the RAC in overseeing individual trials has decreased, but it still serves its federal advisory role in assessing novel biosafety issues. Other forms of stem cell therapy should also proceed with a base of strong pre-clinical data, careful consideration of the clinical setting and approach, as well as well-controlled cell manufacturing and regulatory oversight, to provide maximum safety for subjects and quality of data derived.

Protect subject safety by strict compliance

Any clinical trial with a novel major intervention, such as cell therapy, and especially with subjects with organ dysfunction caused by their disease, can have unexpected and potentially fatal events, either related to the cell product or not. The death of a volunteer subject in a gene therapy trial in 1999 shocked the field and the effects extended throughout much of academic clinical research (Gelsinger and Shamoo, 2008; Wilson, 2009). The response was

to strengthen the quality of clinical trial performance to provide maximum protection to subjects and preserve the integrity of the data they contribute (Wilson, 2009). The field of HSCT began clinical investigations in the 1960's-70's, prior to the establishment of IRB and the other oversight bodies, as best available clinical practice for severe, generally fatal disorders. Since that time, the standards for clinical investigations have been greatly expanded for a much more complex regulatory environment. A typical clinical trial of gene therapy may undergo review by a dozen or more entities, including IRB, IBC, ISPRC, DSMB, NIH RAC, FDA, and one or more funding agencies. HSCT came to full maturity in the U.S. by the development, initiated by members of the ASBMT academic society, of the Foundation for the Accreditation of Cellular Therapy (FACT), which brought uniformity and high standards for cell processing, clinical operations, data management, regulatory management and other clinical trial activities. Commercial cell processing methods and standards have also been developed supporting several cell products that advanced to relatively late stage of investigation (e.g. neural progenitor cells derived from fetal tissue or pluripotent stem cells) and producing a licensed dendritic cell vaccine (the marketed prostate cancer dendritic cell Sipuleucel-T from Dendreon Corp.). This industry has a strong base to produce high quality cell products, but each new cell product type developed brings unique challenges, including details of the cell processing protocol, the release testing, storage, transport, and therapy administration.

Translational research is drug development

It is important to keep in mind that the goal of translational research is to develop a drug that is effective and safe to achieve licensure, be it a cell and/or gene therapy product such as lentiviral-transduced HSCs, iPSC derived myoblasts, dopaminergic neurons, shRNA, etc. This necessitates basic scientists learning fundamental principles of drug development, such as Good Laboratory Practices, Good Manufacturing Practice and Good Clinical practice. An important tool commonly used in drug development is the Target Product Profile (TPP), which sets goals for attributes like: clinical indication, patient population, administration route and schedule, clinical efficacy targets, potential risks, drug quality and testing methods. Early drafting of a TPP can guide subsequent studies to keep focused on the drug development goals.

Trials should be designed to be informative about the cell product

While the primary end-points for early phase trials mostly relate to safety, it should be possible to incorporate secondary end-points for efficacy and exploratory end-points for biomarkers that can be assessed for potential suitability as eventual primary end-points for drug approval. In the clinical trials we have done testing new vectors, there has been a primary end-point assessing safety, which is typical for a Phase I study, but also secondary end-points assessing efficacy. For ADA SCID, this has involved safety assessments by documentation of clinical adverse events, as well as ensuring absence of replication-competent viral vector emergence and absence of vector-driven clonal expansion. Efficacy assessments involved measuring expression of ADA enzyme activity in mature blood cells, quantifying engraftment of gene-modified stem cells by measuring vector copy number in cells by quantitative PCR, and performing standard clinical tests of immune function, as well as recording clinical health.

In some instances, especially with orphan diseases, non-traditional pathways of clinical trials may be accepted by regulators, with even small trials used as pivotal for registration, assuming they were done with appropriate design and rigor. The EMA approval of Strimvelis for ADA SCID was based on a single center's Phase I/II clinical data involving 12 patients which served as a pivotal clinical trial.

Get the most from pre-clinical studies

The other major serious complication in the gene therapy field was the development of leukemia in subjects in several primary immune deficiency trials from insertional oncogenesis by retroviral vectors introduced into HSCs (Braun et al., 2014; Hacein-Bey-Abina et al., 2003; Stein et al., 2010). The relatively high frequency of the development of leukemia in some trials (25–75%) was not predicted by pre-clinical studies. However, pre-clinical models may not detect clinical risks that can occur in patients with much larger absolute cell dosages and longer post-treatment time periods. It may be difficult to test cell therapies by the parameters traditionally applied to drug therapies (pharmacokinetics, biodistribution, toxicity), but these aspects can be often assessed using PCR methods to quantify transgenes or cellular markers, such as human genomes in human cells against the background of murine host genomes.

Nonetheless, pre-clinical studies that are performed should be optimized to provide as much relevant information as possible. Principles of Good Laboratory Practices should be applied whenever possible, even at early stages of discovery and prior to formal IND-enabling studies. These include such key elements of GLP as following a detailed pre-defined plan for the studies, statistical plan, data capture forms, with formal data reporting. Again, even during early phases of product development, it important to include toxicology analysis within efficacy studies to obtain initial information that can be used in the design of definitive studies for IND application. Preliminary proof-of-principles studies can also be used to begin to investigate cell dosages, potential toxicities, as well as disease-modifying activity.

Advancing clinical cell therapies is challenging

Clinical cell therapy requires point-to-point control of the manufacturing process and starting materials (e.g. from skin biopsy to delivery of iPSC-derived somatic cell product, whether it is HSCs or other cell product). The GMP process requires highly trained staff and SOPs, materials specification, batch records, personnel training, in addition to the highly-controlled environment and regulated processes. To characterize the cell product for human administration, it is necessary to define release criteria – identity, purity, potency, and safety. A Certificate of Analysis is completed for each batch of cell product and the testing for each critical attribute is required with full documentation. The analytic testing for aspects of cell quality (e.g. cell counts and viability, immunohistochemistry or flow cytometry, PCR, RNA-SEQ, etc.) should be performed using well-characterized assays, which should be made more robust with advancing stages of investigation.

Conclusion

In conclusion, gene therapy using HSCs has progressed over three decades from ineffectiveness to being able to essentially cure several different disorders. The pathway was not linear, but required multiple iterative bench-to-bedside cycles. It is likely that therapies using other stem cells will also have progress and set-backs. But, because the underlying hypotheses for cellular therapies are so convincing, it is highly likely that multiple novel stem cell-based therapies will be developed. The lessons from the field of HSC gene therapy may provide some guidance for investigators pursuing the translational process.

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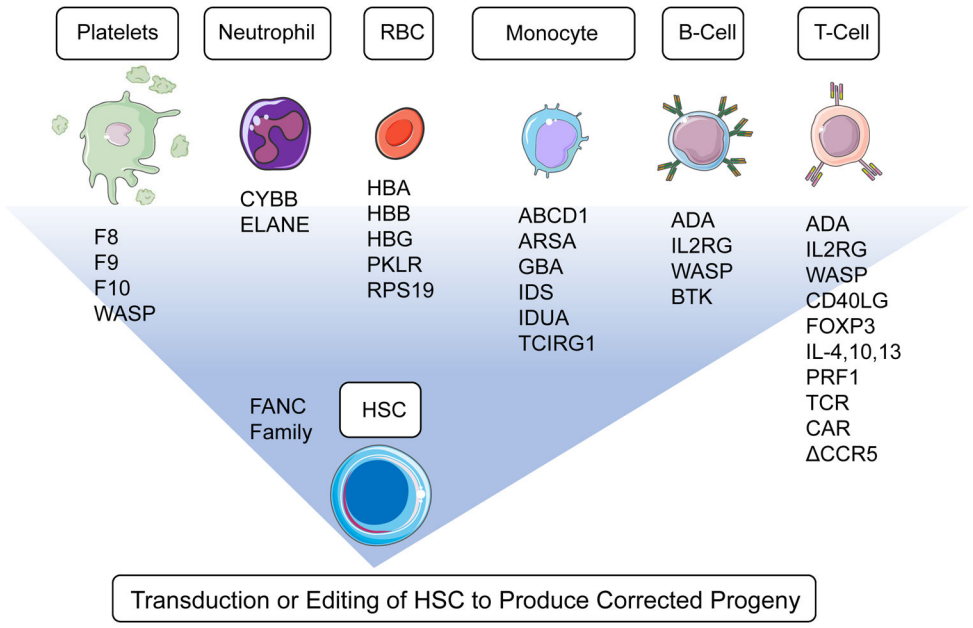

Figure 1. Overview of targets for gene therapy

Hematopoietic stem cells (HSCs) isolated from bone marrow can be modified ex vivo and transferred back to the recipient to produce functional, terminally-differentiated cells. Specific cellular targets and the relevant diseases and genes for gene therapy include the following: **HSCs**: Fanconi Anemia (*FANC A–F*). **Platelets:** Hemophilia A (*Factor VIII (F8)*); Hemophilia B (*Factor IX (F9)*); Factor X deficiency (*Factor X (F10)*); Wiskott-Aldrich Syndrome (Wiskott Aldrich Syndrome Protein (*WASP*)). **Neutrophils**: X-linked Chronic Granulomatous Disease (Cytochrome B-245 Beta Chain (*CYBB*)); Kostmann's Syndrome (Elastase Neutrophil Expressed (*ELANE*)). **Erythrocytes**: Alpha-Thalassemia (Hemoglobin Subunit Alpha (*HBA*)); Beta-Thalassemia and Sickle Cell Disease (Hemoglobin Subunit Beta (*HBB*)); Pyruvate Kinase Deficiency (Pyruvate Kinase, Liver and RBC (*PKLR*)); Diamond-Blackfan Anemia (Ribosomal Protein S19 (*RPS19*)). **Monocytes**: X-linked Adrenoleukodystrophy (ATP Binding Cassette Subfamily D Member 1 (*ABCD1*)); Metachromatic Leukodystrophy (Arylsulfatase A (*ARSA*)); Gaucher disease (Glucosylceramidase Beta (*GBA*)); Hunter Syndrome (Iduronate 2-Sulfatase (*IDS*)); Mucopolysaccharidosis type I (Iduronidase, Alpha-L (*IDUA*)); Osteopetrosis (T-Cell Immune Regulator 1 (*TCIRG1*)). **B Cells:** Adenosine deaminase (ADA)-deficient Severe Combined Immunodeficiency (Adenosine Deaminase (*ADA*)); X-linked severe combined immunodeficiency (Interleukin 2 Receptor Subunit Gamma (*IL2RG*)); Wiskott-Aldrich Syndrome (Wiskott Aldrich Syndrome Protein (*WASP*)); X-linked agammaglobulinemia (Bruton's Tyrosine Kinase (*BTK*)). **T Cells:** Adenosine Deaminase (ADA)-deficient Severe Combined Immunodeficiency (*ADA*); X-linked severe combined immunodeficiency (*IL2RG*); Wiskott-Aldrich Syndrome Protein (*WASP*); X-linked Hyper IgM syndrome (CD40 Ligand (*CD40LG*)); IPEX Syndrome (Forkhead Box P3 (*FOXP3*)); Early Onset Inflammatory \Disease (Interleukin 4, 10, 13 (IL-4, 10, 13));Hemophagocytic Lymphohistiocytosis (Perforin 1 (*PRF1*)); Cancer (Artificial T cell receptors (TCR), Cancer;

Chimeric Antigen Receptor (CAR)); Human immunodeficiency virus (C-C Motif Chemokine Receptor 5 (*CCR5*)).

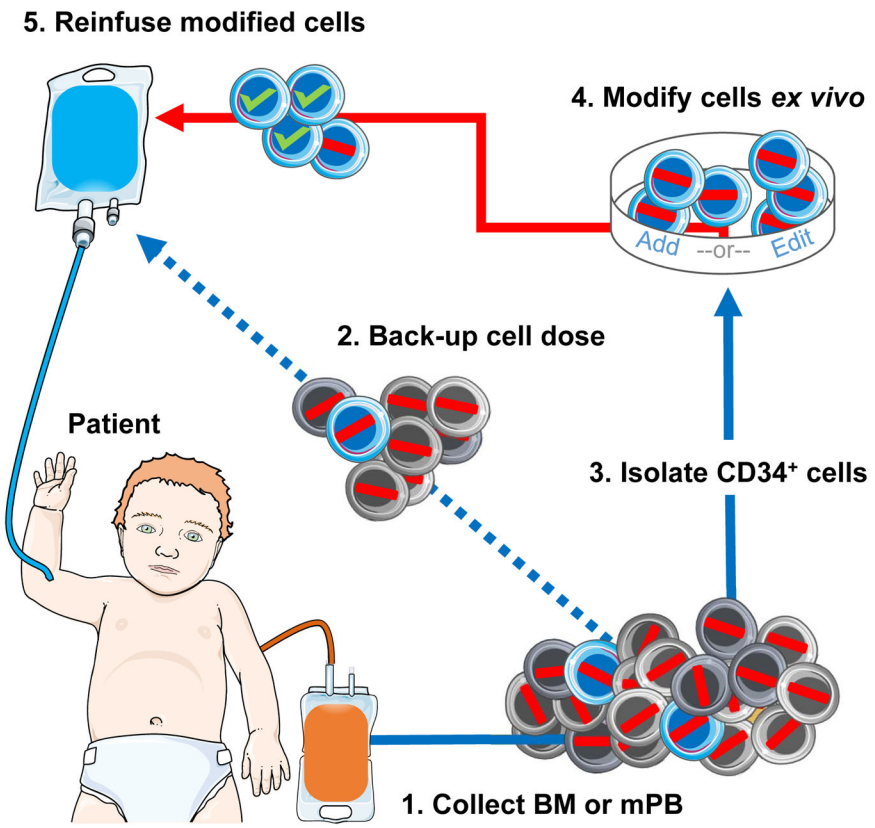

Figure 2. Autologous hematopoietic stem cell transplantation combined with gene addition or editing

(1) Bone marrow (BM) or mobilized peripheral blood (mPB) cells are collected from the patient (red line represents a disease-causing mutation). Typically, 15–20ml of BM/Kg is an acceptable harvest target. While collecting HSCs by mobilization and apheresis is less invasive than BM aspiration, infants have small blood volumes making leukapheresis challenging. Failure to harvest adequate cell numbers can prevent therapy. (2) Modification of HSCs may reduce stem cell capacity. A back-up cell dose of non-modified cells is apportioned to restore native hematopoiesis in the event of graft failure. (3) CD34+ cells are isolated in a GMP-compliant, closed system. Purification of HSCs may reduce total cell number as CD34+ HSCs represent less than one percent of total cells. Alternatively, a CD34+/CD38− enrichment strategy may be employed to further purify HSCs and lower the amount vector required for modification. CD34+ cells may be pre-stimulated ex vivo for 1–3 days prior to modification, depending on the protocol. (4) Gene modification of HSCs must be permanent so as to be passed down to all progeny. Cells are modified by either a viral vector to add a gene (typically requires high concentration vector), or targeted nucleases with/without a donor template to disrupt, correct, or insert a gene. After ex vivo modification, the cell product undergoes release testing to assess purity, identity, safety, potency (transduction/editing efficiency), and other characteristics. If the modification strategy requires selection of corrected cells, low cell yield may prevent transplantation. (5) Prior to receiving the cell product, the patient undergoes conditioning to “make space” for engraftment of modified HSCs (green check represents successful modification of a disease-

causing gene). Modified cells may be reinfused fresh or cryopreserved for delivery at a later time. While high-levels of cytoreductive agents may be toxic, inadequate conditioning may result in poor engraftment.

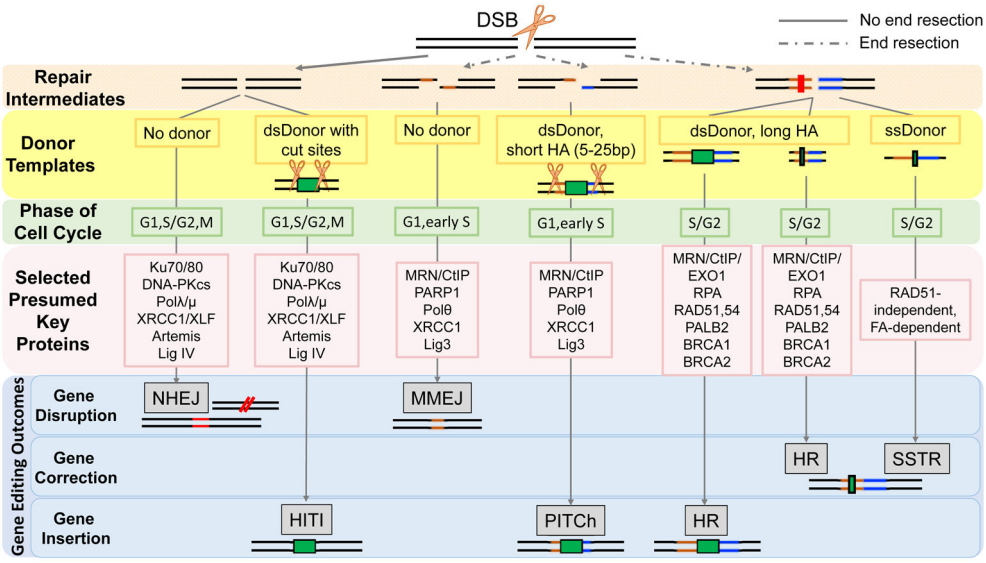

Figure 3. Summary of Gene Editing Pathways

Double stranded break (DSB) is induced by a targeted nuclease (represented by scissors). DSB ends may or may not be resected (dashed or solid line, respectively),. The ultimate gene editing outcome (light blue boxes on the bottom) depends on several factors: the type of donor template provided (yellow box), the phase of cell cycle (light green box) and the presumed DNA repair proteins available (pink box). Gray boxes indicate the names of the repair mechanisms. It should be noted that the figure illustrates the common pathways described to date, however modification of DNA repair pathways and their utilization for gene editing purposes is an area of active research. (From left to right). A DSB with no end resection and no donor available is likely to result in insertions and deletions (indels) and lead to gene disruption via the non-homologous end joining (NHEJ) pathway. NHEJ may occur in any phase of cell cycle. Exogenously providing a double-stranded donor (dsDonor), which contains nuclease cut sites (scissors) around the gene of interest (green rectangle), may result in homology-independent targeted integration (HITI). The presence of microhomology on opposite strands of DNA around the cut site may result in gene disruption via the microhomology-mediated end joining (MMEJ) pathway. A recently reported method of gene integration, termed precise integration into target chromosome (PITCh), utilizes MMEJ machinery to integrate a gene of interest, which is provided by dsDonor with short homology arms (HA) to the DNA (HA are highlighted in orange and blue). The three pathways on the right are generally only active in S/G2 phases of cell cycle and may be used to correct a single nucleotide mutation in the DNA (represented by a red line). Exogenously providing a dsDonor with long homology arms may lead to either gene integration or gene correction via homologous recombination (HR) mechanism, depending on the length of the donor template. A new type of repair mechanism for gene correction was recently described, termed single stranded template repair (SSTR). Although resulting in the same outcome as HR-mediated gene correction, SSTR is presumed to utilize the Fanconi Anemia (FA) pathway and be RAD-51 independent.

Table 1

Genetic diseases of blood cells and the transplantation modalities that have been applied clinically as therapies or are in pre-clinical development.

Category of Disease	Specific Conditions	Transplantation Modalities Applied			
		Allogeneic HSCT	γ-Retroviral Gene Therapy	Lentiviral Gene Therapy	Genome Editing
Primary Immune Deficiencies	ADA-Deficient Severe Combined Immune Deficiency	+	+	+	
	X-linked Severe Combined Immune Deficiency	+	+	+	Pre-clinical
	Other Genetic Forms of SCID (Artemis, Rag1/2).	+		Pre-clinical	
	Wiskott-Aldrich Syndrome	+	+	+	Pre-clinical
	Chronic Granulomatous Disease	+	+	+	Pre-clinical
	Leukocyte Adhesion Deficiency	+	+	Pre-clinical	
	Hemophagocytic Lymphohistiocytosis	+		Pre-clinical	
	X-linked Hyper IgM Syndrome	+			Pre-clinical
	X-linked Lymphoproliferative Disease	+			
	X-linked Agammaglobulinemia	**Few**			Pre-clinical
	Common Variable Immunodeficiency	Heterogeneous Genetic Etiologies, Often Unknown. May Require Editing			
Hemoglobinopathies	Sickle Cell Disease	+		+	Pre-clinical
	β-thalassemia	+		+	Pre-clinical
Storage and Metabolic Disorders	Gaucher Disease and other lipidoses	+	+		
	Mucopolysaccharidoses (I-VII)	+		+	
	X-linked Adrenoleukodystrophy	+		+ Recent Phase II/III	
	Metachromatic Leukodystrophy	+		+	
	Osteopetrosis	+		Pre-clinical	
Congenital Cytopenias and Stem Cell Defects	Fanconi's Anemia	+	+	+	Pre-clinical
	Schwachman-Diamond Syndrome	+		Pre-clinical	Pre-clinical
	Kostmann's Syndrome	+			

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