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Review

Genetic engineering meets hematopoietic stem cell biology for next-generation gene therapy

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https://doi.org/10.1016/j.stem.2023.04.014

SUMMARY

The growing clinical success of hematopoietic stem/progenitor cell (HSPC) gene therapy (GT) relies on the development of viral vectors as portable "Trojan horses" for safe and efficient gene transfer. The recent advent of novel technologies enabling site-specific gene editing is broadening the scope and means of GT, paving the way to more precise genetic engineering and expanding the spectrum of diseases amenable to HSPC-GT. Here, we provide an overview of state-of-the-art and prospective developments of the HSPC-GT field, highlighting how advances in biological characterization and manipulation of HSPCs will enable the design of the next generation of these transforming therapeutics.

INTRODUCTION

Ex vivo hematopoietic stem/progenitor cell (HSPC) gene therapy (GT) consists of the infusion of genetically modified cells that reconstitute the hematopoietic system with their engineered progeny and may rescue a disease pathophysiology up to a cure. HSPC-GT entails a multi-step process starting from the harvest of HSPCs from the patient or, more rarely, an immunologically matched healthy donor; followed by their *ex vivo* culture and genetic modification, whether achieved by gene transfer or gene editing (GE); and finally infusion into the patient, most often after administration of a conditioning regimen to allow efficient engraftment of modified cells (Figure 1).

Because conditioning depletes the resident HSPCs from the bone marrow (BM) to make space for the infused cells, it establishes a transient myelosuppression exposing the patient to the risk of infections and hemorrhages, as it occurs after autologous or allogenic HSPC transplantation (HSCT). The faster the hematopoietic recovery, the safer the treatment and the lower its morbidity.[1] Once full reconstitution has been achieved, long-term follow-up monitors stability of the transduced graft and its therapeutic benefits as well as the potential emergence of delayed adverse effects that can be traced back to the procedure.

Pioneering clinical trials of HSPC-GT were conducted on patients affected by primary immunodeficiencies using gene transfer vectors derived from murine gamma-retroviruses (γRVs).[2–5] Despite the low efficiency of gene transfer into human HSPCs, these studies provided early evidence of the substantial therapeutic benefit that can be achieved upon engraftment of even a small fraction of gene-corrected HSCs (i.e., the rare subset of HSPCs endowed with long-term self-renewing and repopulating capacity) and led to the market approval of the first *ex vivo* HSPC-GT.[6] These studies, however, also highlighted the Achilles' heel of the strategy, as some patients later developed leukemia originating from HSPCs carrying vector insertion next to a cellular proto-oncogene, leading to its overexpression and triggering its transforming potential.[7] The ensuing quest for more efficient and safer gene transfer vectors was met by the advent of pseudotyped lentiviral vectors (LVs),[8] which captured the proficiency of the Human Immunodeficiency Virus (HIV) at delivering its genetic cargo into the nucleus.

CURRENT STAGE OF CLINICAL DEVELOPMENT OF LENTIVIRAL HSPC-GT

The availability of a highly efficient gene transfer vector system has led to broader application of HSPC-GT in several diseases, including primary immunodeficiencies,[9–14] metabolic diseases,[15–18] hemoglobinopathies,[19–21] and BM failures.[22] As of 2020, four hundred patients had received HSPC-GT for the treatment of 14 diseases; 279 thereof had been treated with LVs in 34 trials.[23] Most LV-treated patients reported a substantial and sustained benefit from HSPC-GT thanks to high levels of engraftment with gene-corrected HSPCs. Current numbers are likely to be higher, considering that HSPC-GT products are now available on the market (for β-thalassemia [BThal] and cerebral adrenoleukodystrophy [cALD] in the USA and metachromatic leukodystrophy [MLD] in the EU) and other ones are in advanced stages of clinical development.

Autologous HSPC-GT may eventually position itself as first-line treatment for many monogenic diseases currently amenable to allogenic HSCT. Contrary to the immunological constraints of allogenic HSCT, HSPC-GT is available to every patient and does not entail the risk of developing graft-versus-host disease, which represents a major cause of morbidity and lethality. Given that partial chimerism with functional cells can be easily established with autologous gene-modified HSPCs, lower-intensity non-myeloablative conditioning regimens and emerging chemotherapy-free strategies sparing off-target tissues[24–26] might be successfully employed to further reduce risks and morbidity in

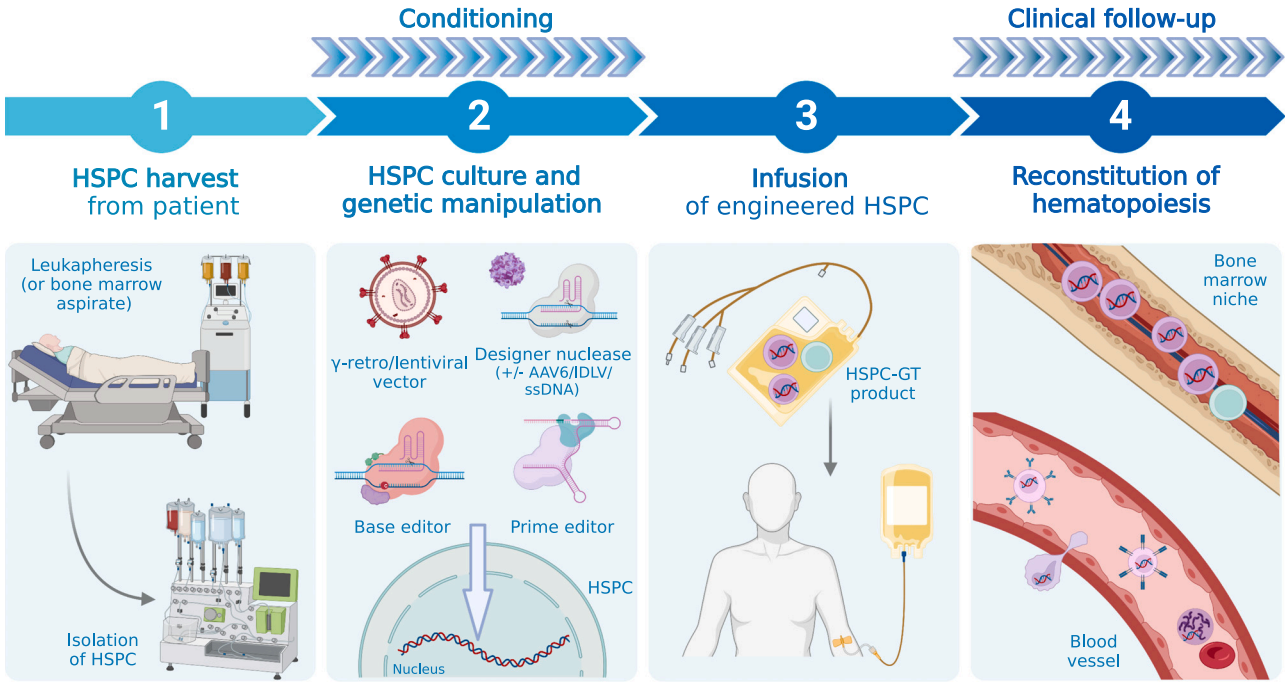

Figure 1. Representation of the HSPC-GT process
AAV6, adeno-associated vector serotype 6; IDLV, integrase defective lentiviral vector; ssDNA, naked single-stranded DNA.

settings where correction of a fraction of HSCs provides the full benefit of the treatment.

Intriguingly, HSPC-GT has been shown to surpass the benefits of allogenic HSCT in some lysosomal storage diseases, like MLD, because of the possibility to establish supra-normal expression of the functional enzyme in the transduced HSPC progeny, resulting in increased clearance of the stored toxic products and bioavailability of functional enzymes for the cross-correction of tissue resident cells.[27] This paradigm may be applied to an increasing number of metabolic diseases.[15–18,28] Applications of HSPC-GT may extend outside of the field of genetic diseases and provide a new platform for targeted delivery of biotherapeutics to disease sites, such as tumors, by combining tissue-specific homing of selected HSPC progeny with advanced engineering of vector design for lineage- and context-specific transgene expression.[29–31] Similarly, microglia-mediated gene delivery may be explored for the treatment of neuroinflammatory and degenerative diseases.

γRVs and LVs integrate semi-randomly genome-wide,[32] and vector Integration Sites (ISs) are distinctive genetic marks of each genetically modified cell and its progeny. Retrieval of ISs using powerful PCR-based techniques coupled to Next Generation Sequencing (NGS) and computational analyses enables detection, output tracking, and quantification of an abundance of thousands of clones from circulating blood cell types, tissues, or plasma serially harvested after GT.[33–41]

Clonal tracking analyses in LV-based HSPC-GT clinical trials have revealed highly polyclonal and multilineage reconstitution of the hematopoietic graft without signs of expanding or -dominant clones over several years of follow-up in most subjects.[12,15,16,19,27,33–37] Longitudinal analyses of the clonal repertoire from early to late phases of hematopoietic reconstitution showed that hematopoiesis is mainly sustained early after transplant by short-lived clones with output in one or few lineages,[38] which are exhausted over time and are replaced by more stable and multipotent progenitors bearing some of the expected features of HSCs. Furthermore, clonal tracking analyses allow the investigation of the dynamics and hierarchical relationships of progenitor subsets,[39] such as evaluation of their heterogeneity in the commitment to differentiate toward specific lineages and the presence of lineage-specific selective advantage in certain diseases.[36]

Further studies may shed light on stability and long-term resilience of the engineered hematopoietic graft and whether the process of *ex vivo* genetic manipulation and/or the replication stress sustained upon hematopoietic reconstitution may induce excess accrual of mutations and endanger a risk of premature drift toward clonal hematopoiesis.[40] Moreover, insights into the factors regulating the choice between activation and latency among the infused HSCs and the establishment of a dormant reservoir of engineered HSCs to provide a response to stress conditions are lacking and remain invisible to current clonal tracking studies but would be important to further perfect transplantation strategies.

Additional platforms for gene addition have been developed from naturally occurring and engineered DNA or RNA transposases on the premise of lower costs and potential advantages from the non-viral origin and integration site preferences.[41,42] Currently, however, their clinical development has been mostly confined to lymphocyte engineering.[43] Further studies exploring the possibility to engineer them for site-specific integration may unleash the full potential of these platforms.

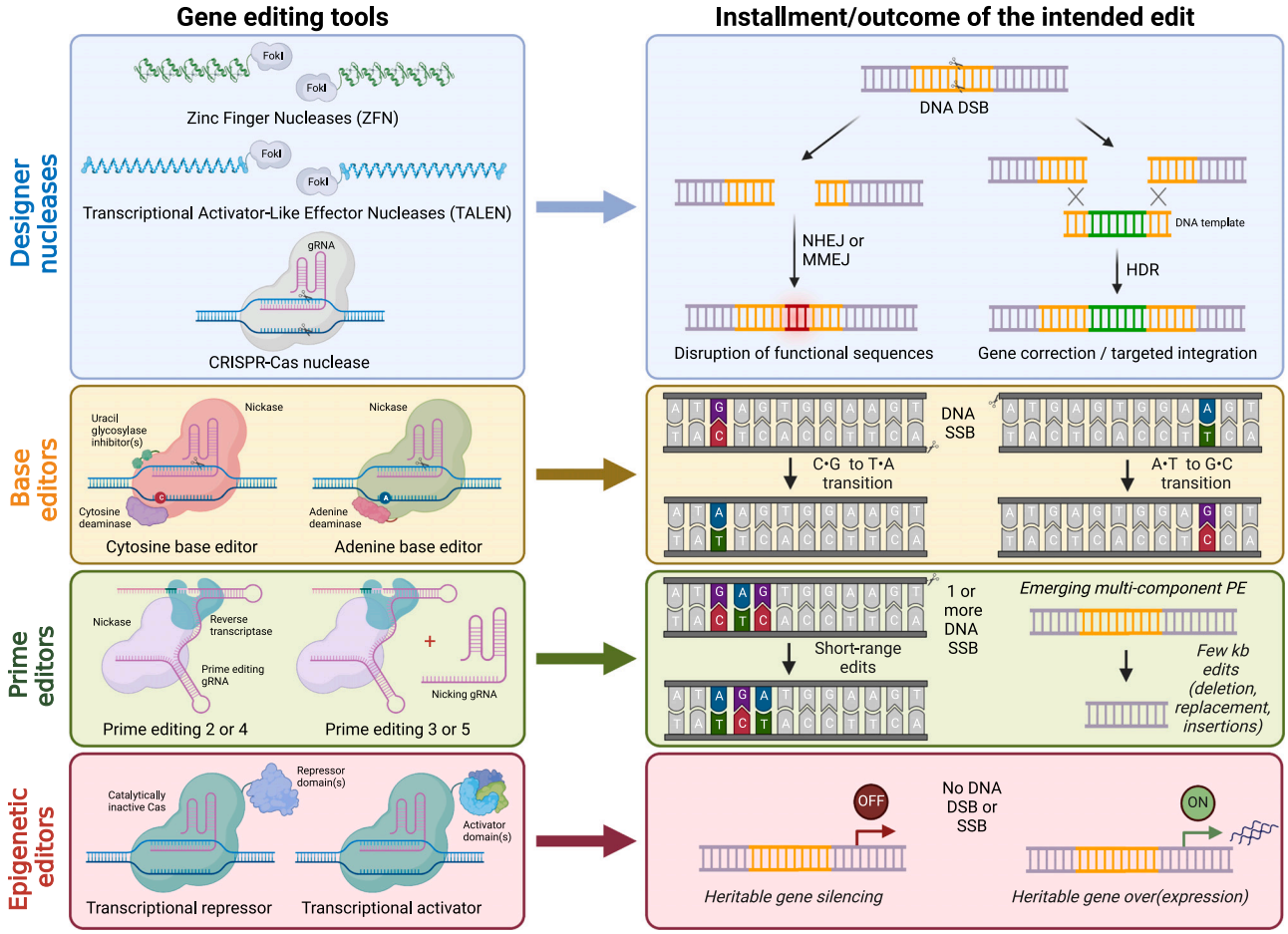

Figure 2. The gene editing tools and the intended outcomes of their activity
Italic descriptions mean that reports showing these outcomes in human HSPCs are currently lacking.

STATE-OF-THE-ART AND EMERGING GENE EDITING PLATFORMS

Designer nuclease-based editing

In the last two decades, the GT toolbox has been enriched with several new programmable chimeric molecules enabling site-specific gene editing (Figure 2).[44] Designer nucleases, such as Zinc Finger (ZFN), Transcriptional Activator-Like Effector (TALEN), megaTAL, and CRISPR-Cas nucleases, are composed of a customizable DNA binding module (either consisting of a protein domain or a guide RNA [gRNA]) and a catalytically active domain triggering DNA double-strand break (DSB) formation upon successful recognition of the target sequence by the binding module. The DNA DSB engages the DNA repair pathways that ultimately result in permanent installment of the intended genetic modification of the target site.[45] Faithful reconstitution of the native sequence is not a favored outcome, as it is subject to another round of cutting by the nuclease. Instead, ligation of DNA ends often exploits the error-prone non-homologous end-joining (NHEJ) and other alternative pathways (e.g., microhomology-mediated end-joining [MMEJ]), introducing small nucleotide insertions and deletions (indels) at the target site that can be expected to compromise recognition by the DNA binding module.

Cells engaged in S/G2 cycle phases may also repair the DNA DSB by high-fidelity homology-driven repair (HDR) in presence of a DNA bearing sequence homology to the cut site. HDR may be exploited to integrate an arbitrary sequence in a precise genomic location, thus overcoming the main limitation of integrating vectors. Thus, exogenous DNA templates carrying the intended new sequence to be pasted into the locus must be provided for HDR editing. According to the size of the intended edit, this can be accomplished with naked single- or double-stranded DNA (ss/dsDNA)[46,47] or by using a viral vector, such as Adeno-Associated Vector 6 (AAV6)[48] or Integrase-Defective LV (IDLV).[49,50]

Eukaryotic cells deploy a composite DNA damage response (DDR) to deal with DNA DSBs induced by exposure to radiations or chemicals.[45] These pathways scan for free DNA ends and converge on the tumor suppressor p53, which slows down cell proliferation while promoting attempts at repairing the DNA lesions and ultimately instructs cell fate among survival, differentiation, senescence, or apoptosis depending on the damage burden and the success or failure of DNA repair.[45] Similarly, one or few nuclease-induced DNA DSBs can trigger p53-dependent DDR in HSPCs, which decreases their reconstitution potential and graft clonal diversity in xenograft models.[51] As the DNA

DSB load increases, such as in the presence of nucleases with poorer specificity, the DDR burden becomes unsustainable, leading to the establishment of inflammatory transcriptional programs, protracted proliferation arrest, or apoptosis and severe impairment of HSPC clonogenic capacity. Because the DDR triggered by DNA DSB may cumulate with that induced by additional editing components (such as the DNA template[51–53]) and *ex vivo* culture per se, minimizing its induction and dampening the downstream consequences by optimizing nuclease specificity and transiently inhibiting p53,[51,54] respectively, might be considered for therapeutic GE.

Engineering of the designer nucleases and optimization of their delivery enabled highly efficient editing of several target sites, reaching nearly full NHEJ-mediated disruption of functional sequences, such as open reading frames and regulatory elements, in human HSPCs.[55,56] Of note, targeted gene knockout/knockdown by disruption of a coding sequence is an outcome precluded to integrating vectors (with few exceptions[21]). Conversely, since long-term repopulating HSCs rarely enter and slowly progress into the cell cycle, targeted integration of long-range therapeutic sequences by HDR is disfavored. Despite the considerable efforts in enhancing its efficiency by optimizing culture conditions,[49,57,58] promoting cell-cycle progression and/or favoring HDR,[54,59–61] and choosing the most appropriate delivery vehicle for the DNA template, HDR editing remains challenging.[46,47,50,52,53] Most promising data in xenograft models show that <30% of long-term engrafting HSCs can be edited by HDR using viral template delivery in the absence of selective advantage or enrichment of modified cells,[54,58,62] which currently constrains prospective HDR application to diseases where therapeutic efficacy and favorable risk/benefit balance can be attained by correcting a minority of the cells. Recent advancements in the design, quality, and delivery of long non-viral DNA templates (ssDNA, dsDNA, and mixed ss/ds DNA carrying Cas recognition sites) enabled promising results in terms of editing efficiency in some differentiated hematopoietic cells, but <10% in bulk HSPCs *in vitro.*[47,63] Of note, when an HDR strategy targets an exon, such as to correct a mutation or to knock in a functional complementary DNA (cDNA) correcting all downstream mutations, it is also relevant to consider the potential adverse effect of NHEJ/MMEJ on the remaining non-HDR edited alleles, whose genetic inactivation may convert dysfunction to loss of function, such as in sickle cell disease (SCD) or in haploinsufficiency disorders such as GATA2 deficiency, RUNX1 deficiency, and Diamond-Blackfan anemia. Due to these challenges, HDR-mediated gene editing lags behind NHEJ-mediated disruption in terms of clinical development, with the latter reporting promising and reproducible efficacy in recent clinical trials for hemoglobinopathies[64] and expected to reach marketing authorization in the upcoming years.

Nickase-based base and prime editing

Base and prime editors (BEs/PEs) are more recent genome engineering tools composed of a sequence-specific DNA binding and nicking module (i.e., delivering a single-strand DNA break [SSB]), typically derived from Cas, fused to constitutively active enzymatic domains, such as deaminases or reverse transcriptases, bearing specific activities on nucleic acids.[65] Of note, the activity of some of these editors depends on or is highly improved by simultaneous inhibition of endogenous repair pathways (e.g., the base excision or the mismatch repair) to reach therapeutically relevant editing efficiencies.[66–68] BEs/PEs can generate more homogeneous genetic outcomes at the target site compared to nuclease-based approaches and can bypass the requirement for DNA DSB and restriction of cell-cycle phases. Their application, however, is mostly limited to installment of short-range edits (A•T>G•C transition by adenine BEs, C•G>T•A transition by cytosine BEs, or all possible insertions/deletions/substitution by PEs) and requires the design of mutation-specific gRNAs when aiming for targeted gene correction, constraining their use outside of diseases for which mutation-specific correction is sustainable for drug development. Recent advancements of the PE technology may enable the installment of longer edits—either deletions, replacements, or insertions—by concurrently delivering pairs of prime editing gRNAs (pegRNAs) or by introducing short target sequences acting as substrates for a successive round of DNA manipulation mediated by site-specific recombinases or integrases.[69–72]

Adenine BEs have reached tolerated and therapeutically relevant editing in human HSPCs,[73–76] prompting clinical investigation of this technology for the treatment of hemoglobinopathies (NCT05456880). Conversely, cytidine BEs have shown lower efficiency and little application in HSPCs so far.[77] Short-range PE has been recently reported in an SCD mouse model by forcing *in vivo* selection of prime-edited cells and at low efficiency in human HSPCs *in vitro.*[78] Furthermore, the feasibility of successfully and safely applying the emergent multi-component PE versions requiring concurrent or sequential steps of complex genetic engineering is questionable and still remains to be demonstrated in HSPCs.

Finally, the possibility to stably switch off/on expression of a target gene by modifying its local epigenetic features also emerged as a DNA break and mutation-free approach. Hit-and-run delivery of a combination of DNA methyltransferase and transcriptional repressor coupled with a sequence-specific DNA targeting domain can instruct stable repression of a targeted locus with high specificity and tolerability in eukaryotic cell lines.[79,80] The application of this platform to relevant primary target cells is being explored to provide an alternative for less harmful and multiplexed engineering.

Emerging editing platforms and prospective improvements

All editing platforms undergo constant evolution toward higher efficiency, specificity, and genome accessibility by discovery of new enzymes and generation of novel variants through rational design and forced evolution.[81–90] Researchers can now choose from an ever-growing armamentarium of editing tools with different breadths of genomic coverage and distinct on-/off-target activities. Some efforts have also been made to enhance specificity and efficiency of DNA/RNA transposons from different species;[91–94] however, they have only reached proof-of-principle in prokaryotes or eukaryotic cell lines. At the present time, HDR editing remains the only viable option for long-range editing and targeted integration of full expression cassettes in human HSPCs, despite its complexity and the limitations of the process.

Ex vivo delivery of editors into HSPCs is mostly achieved by electroporation of their *in vitro* synthesized RNA or

pre-assembled ribonucleoprotein complexes (RNPs) in the case of RNA-guided Cas-based editors.[95–97] This procedure allows robust pulses of expression, reaching high enough cellular concentration to allow efficient processing of the intended genomic target but limiting prolonged and iterative action on on-/off-target sites. Alternative delivery methods, such as lipid- (LNPs) or polyplex-based nanoparticles encapsulating RNA/RNPs, are being explored with the promise of lower toxicity and improved versatility.[98]

GENOTOXIC RISKS OF HSPC-GT

Modification of the human genome comes with an intrinsic genotoxic risk. Vector-mediated insertional mutagenesis and undesired on-/off-target editing activity represent possible genotoxic mechanisms leading to expansion of a clone bearing growth-promoting mutations and paving the way to subsequent accumulation of synergistic mutations, eventually leading to leukemia. This sequence of events may occur in different circumstances, such as aging or exposure to mutagenic drugs, and manifest with variable time courses. Vector insertion and undesired GE events may represent the first trigger, with the likelihood and timing of further progression to transformation being dependent on stochastic and disease-related factors or occurring on a pre-existing clone, accelerating its evolution to full-blown malignancy. The proliferative demand of regenerative hematopoiesis in conditioned patients may favor selection and expansion of clones bearing gain-of-function mutations as well as result in replication stress leading to further accumulation of mutations impacting fitness, long-term stability, and leukemogenesis risk of the cell graft. While similar conditions apply in conventional HSCT, the possible impact of *ex vivo* manipulation on the total amount and quality of infused HSPCs may aggravate this risk. Ongoing studies tracking the rate of accumulation of somatic mutations in hematopoietic cells upon HSPC-GT will provide insights and compare it to HSCT.[99–101]

Risks associated with gene transfer

Because of the high numbers of HSPCs receiving one or more vector insertions semi-randomly distributed across the genome, it is expected that the integrome of an HSPC-GT product nearly covers the accessible genome, including proto-oncogenes and tumor suppressor genes. The genotoxic risk of such insertions depends on whether they cause alterations in the expression level and/or messenger RNA (mRNA) structure of the targeted genes, resulting in activation of the oncogene transforming potential or inactivation of the tumor suppressor gene. The likelihood that an IS deregulates transcription of nearby genes depends on multiple factors. *In vitro* cell immortalization assays[102–104] and *in vivo* oncogenicity studies in sensitized tumor-prone mouse models[105–107] showed that vector design is the most important factor impacting genotoxicity. γRVs and LVs with transcriptionally active Long Terminal Repeats (LTRs) show a higher risk of genotoxicity, as they can proficiently alter expression of the targeted genes by aberrant transcription originating from LTRs or activation of endogenous promoters by interaction with the strong vector LTR enhancers. A preferred vector design thus enforces deletion of transcriptionally active sequences from both LTRs upon reverse transcription and integration of the proviral form (Self-Inactivating [SIN] vectors)[108] and has proven significantly less genotoxic than earlier versions in preclinical genotoxicity assays[102–107] and several clinical trials.[23] However, SIN vectors must comprise an internal promoter to express the transgene, preferably taken from a cellular gene with either housekeeping function and moderate ubiquitous expression or lineage-specific expression, both of which reduce the potential for enhancing transcription of nearby genes in HSPCs. In addition, while both γRVs and LVs preferentially integrate into the open chromatin of expressed genes, they show distinct biases for some genomic features (i.e., the preference of γRV for insertion near active promoters),[32] affecting the risk of enhancer-mediated genotoxicity.

As SIN vectors may harbor cryptic splice sites, they may occasionally alter the mRNA structure of a host gene within which they have been inserted and induce premature termination or interfere with splicing.[109–111] While the genotoxic risk of such mechanisms is likely low for commonly used LVs, its occurrence has been advocated in two different HSPC-GT clinical trials for BThal and X-linked Severe Combined Immunodeficiency (SCID-X1).[11,112,113] In these trials, vector integration triggered HMGA2 overexpression and expansion (but not full transformation) of the clones bearing this IS. In another HSPC-GT developed for cALD, the SIN LV was designed to comprise a strong internal promoter derived from a γRV LTR (MND) to drive robust expression of the transgene product. Long-term follow-up reported clonal expansion and emergence of myelodysplastic syndrome (MDS) in three patients, likely due to insertional activation of the *MECOM* oncogene.[18,114,115] Of note, despite the documented risk of delayed adverse events, the severity of cALD and the substantial benefit provided by HSPC-GT may outweigh the risk of malignancy, according to FDA approval of the therapy. Thus, while the most used LV backbones have shown excellent long-term safety in a growing number of patients and diseases, attention to changes in vector design remains paramount to continue granting it. Incorporation of additional features such as chromatin insulators may alleviate the residual risk of enhancer-mediated transcriptional deregulation near the IS;[106,116] despite its impact on the overall safety and proficiency of the vector, its effect on the regulation of the genomic region harboring the IS must be carefully evaluated, and its relevance in the context of HSPC-GT has yet to be evaluated.

Besides the risk of malignant transformation, vector-mediated oncogene activation in HSPCs could trigger the activation of cellular senescence.[117,118] However, whether treatment-induced senescence may broadly affect hematopoietic reconstitution and functionality across HSPC-GT applications remains to be investigated.

Risks associated with gene editing

In principle, nuclease-based GE strategies allow for higher levels of specificity compared to semi-random integration of viral vectors. Despite the technological advances and the constant amelioration of nuclease specificity, DNA DSBs at genomic on-/off-target sites still represent the major potential source of genotoxicity for clinical applications. Depending on the genomic location, off-target DNA DSB generation may lead to inactivation of tumor suppressor genes, off-target incorporation of the transgene, or chromosomal rearrangements[119–121] (Figure 3). A

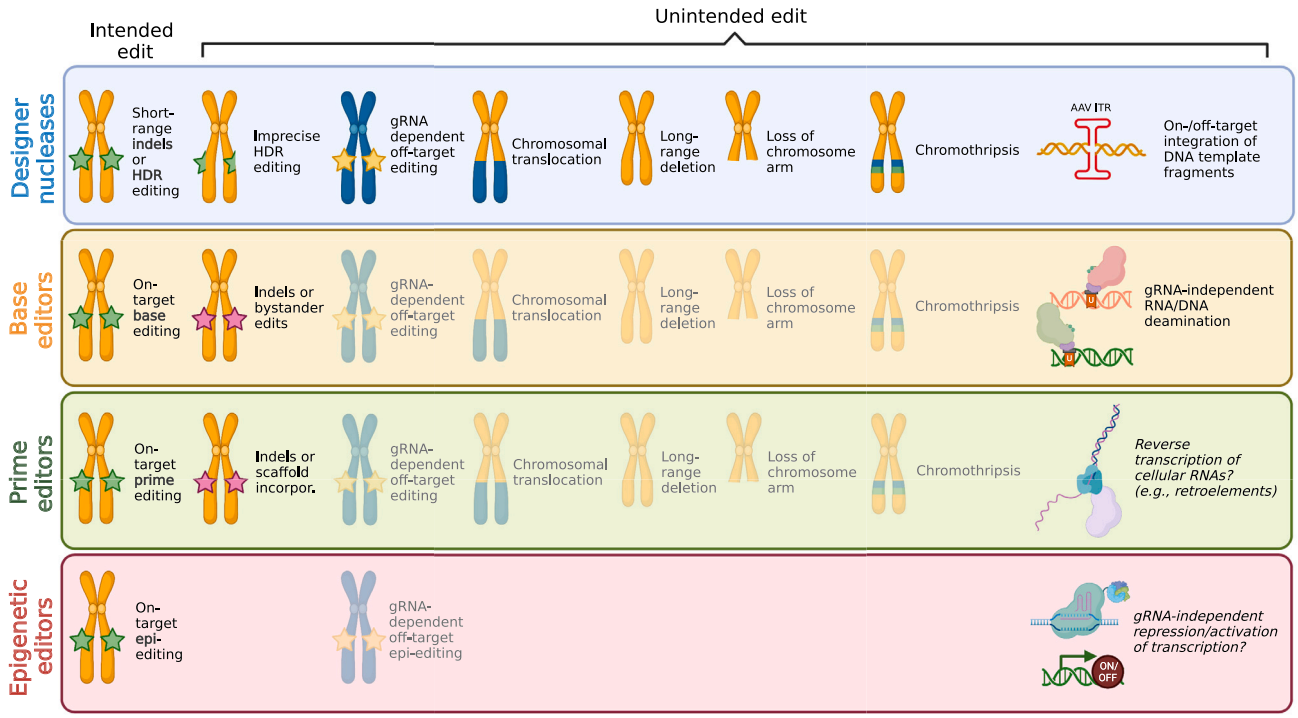

Figure 3. Schematics of the potential genotoxic events occuring at on-/off-target sites upon GE with different platforms

Shadowed drawings and descriptions mean lower likelihood of the editing event to occur compared to other platforms. Italic descriptions with question marks mean that these editing outcomes are only suspected but not yet proven.

growing armamentarium of cell-free and *in cellula* assays as well as *in silico* prediction algorithms were developed to identify putative off-target sites in edited cells.[122,123] Specificity assessment of an editor starts from a thorough bioinformatic analysis verifying the unique occurrence of the target sequence and its most homologous permutations, followed by an experimental analysis usually performed in two steps. The first step aims to identify the most comprehensive panel of putative off-targets in an unbiased and highly sensitive manner, and it is usually performed using two orthogonal techniques, challenging the editor with chromatin/DNA *in vitro* and/or by transfection into human cells. These methods usually yield a high number of putative off-targets, often only partially overlapping among different methods. In this context, several studies are thus aiming to identify the combination of assays and tools providing the highest sensitivity and specificity for off-target site calling while minimizing false positive and false negative rates.[124,125] The calling of putative off-target hits within a validated tumor suppressor gene might kill the candidate nuclease, as biallelic targeting is a realistic possibility when using gene editing tools at variance with gene transfer vectors that may inactivate one allele by random integration but would hardy hit both within the same cell. The next step in the specificity assessment is then to interrogate by targeted deep-sequencing as many top-ranking candidates as feasible in the cell type of interest because specific cellular features may impact the off-target profile.[126] The nuclease delivery method and the duration of editor expression may also influence specificity,[127] and thus the second step of the analysis should be performed in conditions closely recapitulating those intended for manufacturing the therapeutic product. These analyses have a sensitivity threshold ranging between 0.1% and 1% and will be blind to any off-target activity occurring at a lower rate. Because, similarly to gene transfer, a typical HSPC-GT product comprises hundreds of millions of cells, the occurrence of rare events remains possible and uninvestigated, and this uncertainty must be considered when performing risk assessment of any new procedure. Moreover, interindividual variability in the human genome may affect the off-target profile, thus adding another layer of complexity in specificity analyses and off-target validations.[128,129]

While most scientific efforts have been focused on identifying and mitigating nuclease off-target activity by increasing specificity, recent evidence also pointed to a previously unappreciated on-target genotoxicity risk, mainly associated with loss of genetic material. Indeed, Cas cleavage could also generate long-range deletions (from >200 up to several thousand base pairs [bp]) and large insertions ($\geq$50 bp) at the cut site in human cell lines and primary cells, including HSPCs.[50,119,130–135] Moreover, chromosomal translocation events between on- and off-target sites have been shown in human HSPCs and non-human primate (NHP) models, sometimes even persisting *in vivo*.[51,124,136] Sporadic occurrence of extensive rearrangements of one or few targeted chromosomes upon Cas9 cleavage, resulting in the formation of micronuclei, chromosomal bridges, and aberrant nuclear structures, known as chromothripsis, has also been reported.[137,138]

For HDR editing, another factor to take into consideration is the risk of on-/off-target insertion of the DNA template, as shown

for AAV inverted terminal repeats (ITRs), which are trapped in human HSPCs at a relatively high rate, including in long-term engrafting clones in xenografts.[50] While this may often be inconsequential, AAV ITRs exhibit transcription-promoting activity, and AAV integrations have been occasionally associated with carcinogenesis in some animal models.[139–143] In addition, integration of viral vector fragments may happen in randomly occurring DSBs located at sites of slow/altered DNA replication rate, such as chromosomal fragile sites.[144] IDLV as a DNA template appears less prone to fragmentation,[50] albeit not exempt from trapping of its full-length sequence, partially reducing heterogeneity of genetic outcomes and the genotoxic risk of on-/off-site integration due to the presence of SIN LTRs.[108] Instead, the integration profile of ssDNA and dsDNA as HDR templates has not been investigated yet. Overall, the potential issues arising from off-target integration of a DNA template should be taken into account when designing the therapeutic cassette comprised therein.

Some unwanted consequences of nuclease-mediated GE may be mitigated by BEs and PEs (Figure 3), as they aim to conserve the continuity of the DNA at the target site. However, a comprehensive assessment of BE and PE specificity and safety in HSPCs is still missing. Indeed, BEs might raise additional safety concerns arising from the constitutive activity of the deaminase domains that may cause semi-random and gRNA-independent deamination at genomic susceptible sites, such as R-loops, and on the RNA, as shown in cell types different from HSPCs.[145–148] Similarly, further studies are needed to address the putative, but still unproven, potential genotoxic risk of PE due to transient expression of its reverse transcriptase module and eventual inhibition of DNA mismatch repair in PE4 and PE5 systems[66,67] in HSPCs. Furthermore, the SSB generated by the nickase domain of BE may be converted into DSB during DNA replication and eventually result in the adverse consequences discussed above, albeit to a significantly lesser extent compared to designer nucleases. Moreover, indels are introduced at the target site to some extent by the staggered double nicking delivered by PEs (particularly with PE3 and PE5 systems), again raising the risk of DSB generation and its unintended consequences,[149,150] such as tandem duplication, prime editing gRNA scaffold incorporation, long-range insertions/deletions, translocations, and LINE-1 transposable element subfamily integration.[66,70,151–153] Of note, considering the high number of HSPCs to be treated and infused, an even lower frequency of potentially genotoxic editing events might still be problematic when aiming for clinical translation of HSPC GE.

Currently, none of the existing GE platforms fully accomplish the promise of seamless genetic engineering devoid of genotoxic risk in HSPCs. Preclinical assays predicting whether any newly assembled or further evolved GE tool might turn into excessive genotoxic burden and outweigh the expected benefits would be highly relevant. The predictive power of *in vitro* cell immortalization assays and *in vivo* oncogenicity studies in tumor-prone mice is thus limited by the poor flexibility of the mutagenic input of the former and the challenge against a genome different from the human one for the latter, ultimately hampering inference on genotoxic risk related to a designer editor. Aberrant expansion or altered growth/differentiation properties of one or more human HSPC clones in xenograft models from an initially polyclonal product is the most advanced experimental setting able to readout intrinsic genotoxic risk of the procedure due to the accrual of gain-of-function mutations prodromic to cell transformation.[154] Longitudinal tracking of HSPC clones may be pursued by retrieving indels[56] or degenerated molecular identifiers[54,155,156] introduced at the target site by nuclease-mediated editing. In the absence of such a possibility, pre-labeling edited HSPCs with barcoded LVs or reconstructing clonal phylogeny by accumulation of somatic/mitochondrial mutations[157] may be alternative options for preclinical assessment of potential treatment-related adverse effects on clonal outgrowth in a recipient. Clonal tracking studies in NHPs may be important to further validate the impact of novel gene editing technologies on HSPC biology at preclinical level.[56,158]

THE IMPACT OF *EX VIVO* CULTURE ON HSC BIOLOGY

Ex vivo activation is still a prerequisite to reach sufficient levels of HSPC genetic engineering for clinical applications. Culture conditions have been tailored over the past decade to ensure high gene transfer and GE efficiencies and preservation of some long-term repopulating capacity of HSCs upon manipulation.[49,159,160] While γRV gene transfer is strictly dependent on proliferation of the infected cells, LVs bypass this requirement, but activation of HPSCs from the resting condition is required for efficient transduction, usually achieved upon three days of culture in stem-preserving and early active cytokine medium.[161] Recently, transduction enhancers (see "Investigating and overcoming HSPC responses to gene transfer and GE") enable the generation of high proportions of genetically engineered HSPCs by one instead of two cycles of LV transduction, thus shortening time in culture,[15] with the resulting cell products yielding faster hematopoietic reconstitution and fully transduced hematopoietic grafts in patients.[15] Current GE protocols are also designed on three to four days of culture to activate HSPCs[160,162–170] and, in the context of HDR editing, require full progression of the most primitive cells to S/G2 phase of the cell cycle to achieve a sizable fraction of edited cells in long-term grafts.[49,54,57,58] Even if these relatively short culture times may allow no more than one/two cell divisions of the rare HSCs, emerging evidence suggests that they may elicit cellular responses negatively impacting the activity and content of long-term multilineage repopulating progenitors.[160,171,172] While physiologically in a quiescent state, HSCs in culture are forcibly pushed out of their dormant state and may eventually accumulate DNA damage[173,174] and spontaneous mutations or chromosomal abnormalities,[175] ultimately resulting in premature exhaustion and repopulating dysfunctions. Indeed, activated HSPCs produce reactive oxidative species (ROS) and may establish an aged-like phenotype with overt myeloid skewing.[99,176–183] Culture stress may be further exacerbated during genetic engineering because of the p53-mediated DDR activation, overall leading to cell differentiation, loss of engraftment of early progenitors,[171,184] and reduced graft clonality.[54]

Despite all of the above, clonal tracking studies in humans provide evidence that HSCs that have been cultured *ex vivo* for a few days and genetically engineered by viral gene transfer have the potential to robustly engraft and sustain multilineage hematopoietic reconstitution for decades.[1] Consistently, the longer the time

in culture, the higher potential impact on the content of HSCs in the product and consequently on the clonal repertoire of the genetically engineered graft and possibly on their capacity for multilineage output. Whether an oligoclonal input of the hematopoietic graft may constrain its long-term resilience may depend on the capacity of engrafted HSCs to undergo robust symmetric self-renewing divisions *in vivo*, which escape detection by current clonal tracking studies. Intriguingly, reports of spontaneous single clone somatic reversion of some genetic deficiencies leading to sustained mosaicism with functional reconstitution of hematopoietic lineages and significant therapeutic benefit would support this contention.[185–188]

Of note, experimental studies of human HSPCs have been mostly conducted by transplantation experiments in xenograft models,[189,190] which are limited in capturing the full spectrum of HSPC multipotency[191,192] due to the lack of a humanized niche as well as the short time window of analyses. The advent of single-cell omics technologies[181,193] and advanced single-cell-derived colony forming assays[99,194–196] enable comparative characterizations of the properties of bona fide HSCs and committed progenitors; however, none of this emerging biology has been integrated yet into innovative culture systems to capture and preserve HSCs for genetic engineering. The recently developed map of genes and signaling pathways identified during HSC ontogeny in human embryo may uncover additional instructions to keep fully functional HSCs *in vitro*[197–199] and/or enable efficient differentiation of human pluripotent stem cells into bona fide HSPCs.[199–201]

Significant efforts have been directed toward the identification of compounds that could promote expansion of a $CD34^+CD90^+$ population enriched for phenotypic HSCs displaying long-term repopulating activity in limiting dilution *in vivo* assay.[160,162,165,202] The definitive proof that these culture conditions can expand, rather than maintain primitive long-term HSCs while promoting the proliferation of short-term multilineage progenitors, remains to be achieved. Recently, cytokine-free culture conditions have been shown to support robust human HSC expansion *ex vivo*.[203] In addition, attempts to reproduce mechanical and niche-dependent signals may mitigate culture-induced stress responses to improve preservation or achieve successful HSC expansion for genetic engineering.[204] A combination of innovative expansion conditions coupled to multi-omics single cell transcriptional/epigenetic profiling and advanced 3D culture systems may provide a quantitative and qualitative assessment of *ex vivo* cultured HSCs that is predictive of their long-term functionality upon genetic manipulation. These advances may eventually bring into reach the long-sought goal of truly expanding HSCs *ex vivo*, opening the way to the possibility of selecting cells bearing intended genetic edits.

INVESTIGATING AND OVERCOMING HSPC RESPONSES TO GENE TRANSFER AND GE

All gene transfer and GE technologies expose cells to exogenous nucleic acids and/or viral vectors. Innate immunity and nucleic acid sensors might thus affect the cellular response and efficiency of genetic manipulation.[205]

Pattern recognition receptors (PRRs) recognize evolutionarily conserved structures on pathogens (pathogen-associated molecular patterns) within the specific compartments that they patrol. Engagement of Toll-like receptors or the cytosolic nucleic-acid-detecting immune receptors, such as the RIG-I family of helicases (RIG-I, MDA5, and LGP2) and the DNA sensors cGAS, IFI16, or AIM2, will ultimately elicit type-I interferon (IFN)-mediated antiviral responses.[206–208] HSPCs express a variety of these PRRs that enable them to detect and respond to foreign pathogens or endogenous damage.[205] In the context of viral gene transfer, substantial differences have been observed in HSPCs in response to distinct vectors. γRVs, but not LVs nor AAVs, trigger IFN-stimulated antiviral genes.[171] HIV-derived LVs likely benefit from mechanisms of active innate immune evasion that the parental virus evolved to avoid cGAS/STING-dependent sensing of the viral nucleic acids within the cytosol.[209] In the context of GE, exogenous RNA used to express the editor, as well as gRNA, are recognized in the cytosol by the RIG-I pathway and trigger a wave of type I IFN responses.[97,210] Removal of the 5′-triphosphate from gRNA and *in vitro* RNA synthesis using modified nucleotides[211,212] alleviates inflammatory signaling and some of the consequent detrimental responses to GE.

For LVs and AAVs, it is the vector DNA that is sensed within the HSPC nucleus, likely through reverse transcription or replication intermediates, respectively, that mimics the ends of broken DNA and leads to activation of p53-mediated DDR.[50,171] Transient curtailing of vector-mediated activation of these p53-dependent responses significantly improves engraftment and clonality of genetically manipulated HSPCs.[50,51,54] This may be particularly relevant when aiming for HDR editing, given the convergent impact of template viral vector sensing and DNA DSB on DDR.[51–53] Such an adverse response might have contributed to the severe delay in engraftment recently reported for the first SCD patient receiving HSPCs treated by AAV6-mediated HDR editing (https://ir.graphitebio.com/press-releases/detail/84/graphite-bio-announces-voluntary-pause-of-phase-12-cedar). Whereas one may raise concerns for suppressing, albeit transiently and incompletely, the p53-dependent genome integrity keeper and tumor suppressor pathways, this strategy may prevent both an overall clonal loss and the selection of clones bearing or spontaneously acquiring *TP53* genetic inactivation during the manufacturing process.[213–215] Of note, short ssDNA templates have been reported to avoid activation of p53-dependent DDR,[51–53] while little is known about longer ss/dsDNA.

Electroporation, however, may come at the cost of significant toxicity, which may lead to acute loss of viability and transient growth arrest, although followed by recovery of surviving cells.[54] Indeed, electroporation per se may activate DDR, inflammatory responses, transient growth arrest, and apoptosis in a fraction of treated HSPCs.[54] As LNP-based delivery gains more foothold in the field, it will be important to address potential innate immune activation elicited by components of these particles and their nucleic acid or protein cargos in the context of HSPC genetic engineering.

Cellular recognition of exogenous components may also constrain the efficiency of genetic manipulation in human HSPCs. Some antiviral type-I IFN inducible host factors (restriction factors) are highly expressed at steady state in HSPCs.[216] The IFN-inducible antiviral protein IFITM3 was identified as a potent restriction block to LV gene transfer in HSPCs,[217] which can be overcome through molecules such as Cyclosporin H (CsH),[217] Rapamycin,[218] or Resveratrol,[219] significantly enhancing

gene transfer. The potential benefit of transduction enhancers is best shown by the improved clinical success rate of BThal and SCD HSPC-GT achieved by modified gene transfer protocols, including some small molecules increasing LV uptake and integration in HSPCs, such as poloxamers and prostaglandin E2 (PGE2),[35,220] respectively. Of note, the increased transduction levels obtained by these enhancer combinations are much higher when measured *in vitro* in the infused drug product than *in vivo* in xenograft models[221,222] or in cells harvested from HSPC-GT patients.[35,220] This suggests that PGE2 and poloxamers may preferentially enhance transduction of short-term progenitors versus long-term repopulating HSCs, highlighting the challenge of accurately measuring gene transfer in the clinically relevant HSC compartment versus bulk manipulated populations through simple assays amenable for Chemistry, Manufacturing, and Controls (CMC) development. As single-cell technologies become more affordable, analysis of transduction levels within HSCs could also become feasible for drug products, potentially improving their predictive value for long-term clinical efficacy. Significant donor-to-donor variability remains a challenge that does not seem to be overcome by these enhancer combinations. While the determinants of donor variability remain unclear and likely involve several parameters, differential expression of antiviral factors such as IFITM3 or TRIM5 correlates with transduction efficiencies in individual donors.[217,223] CsH may overcome, at least in part, donor variability during HSPC-GT,[217] potentially representing an additional major benefit for the achievement of more standardized medicinal products.

THE IMPACT OF STEM CELL SOURCE, DISEASE BACKGROUND, AND CONDITIONING REGIMEN ON HSPC-GT

Beyond genetic modification, the clinical outcome of HSPC-GT depends on several factors, including stem cell source, patient's clinical condition, dose and quality of the cell product, status of the recipient BM microenvironment, and conditioning regimen.[1,224]

HSPC source and harvest

Cell number and quality of collected HSPCs are critical parameters for ensuring fast hematopoietic recovery and long-term stability of HSPC-GT, also considering the loss upon manipulation and the amount required for quality controls. Mobilized peripheral blood (MPB) HSPCs have become the preferred source for patients undergoing HSPC-GT due to the reduced invasiveness and the higher numbers of HSCs collected, which reduces age-related and individual variability associated with BM HSPC harvest.[19,35,225,226] Mobilization of HSPCs in GT trials is currently based on Granulocyte-colony stimulating factor (G-CSF) administration, usually in combination with the CXCR4 antagonist Plerixafor.[18,19,35] Of note, G-CSF administration is avoided in specific disease settings, such as SCD, because of potential life-threatening complications. In line with HSCT results,[225,227] MPB HSPCs showed faster myeloid reconstitution and increased transduced cell chimerism in the BM, leading to higher clonal composition of the engrafted stem cells and increased correction of mature myeloid populations with respect to patients treated with BM-derived HSPCs.[36,228] Differences in cell state and HSPC composition might explain the distinct behavior *in vivo* of the two sources,[229–231] as the higher proportion and number of myeloid and primitive progenitors in MPB HSPCs correlated with earlier neutrophil recovery and higher transduced cell chimerism in the long term.[228] HSPC collection still presents challenges in certain pathological conditions with low HSC content, heavily pre-treated patients, or low-body-weight pediatric subjects. *In vitro* expansion protocols[162,165,202] are an attractive strategy to address these hurdles.

The impact of disease background

The disease background can impact the source and composition of the HSPC harvest as well as their behavior after transplantation. For instance, in bone marrow failure syndromes, cells are often more fragile and susceptible to culture stress. In other cases, disease features confer selective survival and/or growth advantage to the corrected cells, such as those occurring in Adenosine Deaminase (ADA)-SCID and Wiskott-Aldrich (WAS) for lymphoid cells,[10,13] in WAS for platelets,[13,232] in BThal and SCD for erythroid cells,[233,234] and in Fanconi anemia for HSPCs.[22] In these contexts, therapeutic benefit may be achieved even at low chimerism of transduced HSPCs and with avoidance or reduction of the intensity of the conditioning regimen, as shown for Fanconi anemia HSPC-GTs, in which a limited input of gene-corrected HSPCs infused without conditioning progressively expanded in the BM, eventually rescuing hematopoietic output in the periphery.[22,235,236]

Recent studies highlighted that HSCs and BM niche components are altered in BThal and SCD[237–239] and that targeting the identified defects can rescue the impaired HSC-BM niche crosstalk.[240] Chronic inflammation is a key component in SCD pathophysiology, and increased inflammatory cytokines were reported to alter components of the BM microenvironment, such as osteo-lineage and endothelial cells, in SCD mice.[241,242] Exposure to inflammatory signals and infections triggers the activation of HSCs by acting on cycling properties, DNA damage, and long-term self-renewal[243–245] and can also injure BM niche populations. Impaired HSC function, as well as defective supporting activity by niche components, might be the cause of the low levels of genetically modified HSPCs long-term engrafted in some patients experiencing a lack of clinical benefit in recent HSPC-GT trials,[19,20,35] although the root causes have not been clarified yet. BM niche defects may be further exacerbated by potential damage to hematopoietic and stromal components upon myeloablative conditioning, compromising their supportive capacity. Deeper knowledge of the BM and HSC features in inherited hematological disorders may ameliorate HSPC-GT outcomes by targeting specific niche defects before and/or after transplant and by tailoring conditioning regimens.

Disease background may also impact the risk of insertional mutagenesis, as shown by comparing the incidence of vector-induced leukemias in three early γRV-based HSPC-GT clinical trials for WAS, SCID-X1, and ADA-SCID.[23] While WAS patients developed several myeloid and lymphoid dominant clones in their hematopoietic graft, eventually leading to MDS and/or T-Acute Lymphocytic leukemia (T-ALL), SCID-X1 patients developed only T-ALL or T cell lymphoma with lower incidence, and ADA-SCID patients showed even safer outcomes, with only one patient reported to develop T-ALL in the long-term

follow-up.[23] The specific biological activity of the transgene product and the potential consequences of its absence or de-regulated expression upon HSPC-GT may affect the likelihood of expansion and progression to transformation of clones bearing genotoxic ISs.[246–248] Some diseases may also increase the risk of cell transformation without necessarily involving a causal role of vector insertions. In a recent HSPC-GT clinical trial for SCD, two patients developed MDS and acute myeloid leukemia (AML).[220,249] For both adverse events, the role of vector insertions was eventually dismissed, as the blasts did not contain the vector in one case, and in the second case, vector ISs targeted the *VAMP4* gene, which is unrelated to cancer and was not de-regulated.[250–252] Therefore, it appears that the well-documented increased risk of development of MDS and AML in SCD patients could be the underlying cause of the malignancies observed in HSPC-GT for this disease.[253–255]

Tailoring the conditioning regimen to maximize HSPC engraftment and prevent adaptive immune responses

To achieve sufficient and stable engraftment of gene-corrected HSPCs, patients undergoing HSPC-GT often receive a chemotherapeutic conditioning regimen, whose intensity and type can be tailored to reach a minimal efficacious therapeutic target level while reducing toxicity.[256] Myeloablative doses are necessary when high levels of engraftment are needed; specific tissue targeting, such as the brain, may further constrain the regimen choice to specific drugs. Whereas immune suppression is usually not required due to the autologous cell source, lymphodepletion may still be required to remove pre-existing immune cells reacting against the transgene, such as in patients on enzyme replacement therapy, or to eliminate autoreactive cells in autoimmune diseases.

Introducing a transgene into HSPCs and their progeny may lead to immune response toward neoantigens,[257] especially in individuals with null mutations in the target gene or when the transgene is represented by newly engineered molecules, as host lymphocytes have not been educated to tolerate those specific peptides. Nevertheless, in the context of autologous HSPC-GT, it is expected that lymphocytes deriving from the gene-modified HSPCs undergo canonical negative selection *in vivo* for reactivity against self-antigens, including the transduced neoantigens, ensuring central tolerance. Indeed, in HSPC-GT clinical studies, immune responses to transgenes have rarely occurred,[257] and in those cases, they were transient and did not affect clinical outcomes.[258] Moreover, even in the event of patients carrying pre-existing antibodies to enzyme replacement therapy, such as in mucopolysaccharidosis type 1H, HSPC-GT associated with conditioning was sufficient to induce rapid disappearance of antibodies and tolerance to the transgene.[15] Importantly, HSPC-GT ameliorated or resolved autoimmune manifestations of disease in the case of WAS[12,13] even with partial engraftment of gene-corrected cells.

Non-genotoxic conditioning based on monoclonal antibodies directed against cell surface antigens expressed by all blood cells, such as CD45,[26,97] or by HSPCs, such as CD117,[25,259,260] may become an alternative to chemotherapy to deplete resident stem cells and have indeed demonstrated efficacy in different preclinical models.[261] Beside sparing non-hematopoietic tissues, their non-genotoxic mode of action would also alleviate concerns for mutation accrual in residual HSCs surviving conditioning. Indeed, exposure to conventional conditioning regimens has been proposed to contribute to the development of some hematopoietic malignancies from pre-existing resident HSCs in SCD HSC GT trials and in mixed chimeric SCD allotransplanted patients.[253,262] Anti-CD117 (JSP191) monoclonal antibody is currently exploited in HSCT trials, alone or in combination with chemotherapies, as a conditioning regimen in patients affected by SCID (NCT02963064), Fanconi anemia (NCT04784052), and AML or MDS (NCT04429191), respectively. Early results from an SCID trial showed that 6 out of 12 patients reached >3% donor blood granulocytes at >24 weeks post-HSCT, resulting in clinical improvement including reduced immunoglobulin dependence and antibody responses to vaccines.[263] No treatment-related adverse events were reported. Monoclonal antibodies coupled with toxins or radioisotopes, combining antibody specificity with the toxicity of chemo- and radiotherapies, are under investigation in the clinic[264] (NCT05223699). These antibody-drug conjugates showed efficient depletion of murine and human HSCs in wild-type and hematochimeric mice, respectively, as stand-alone treatments, establishing long-term stable donor chimerism with reduced toxicity as compared to classical radiotherapy.[26,51,261] However, emergent severe toxicity has halted clinical development of one of the above-mentioned antibody-drug conjugates, raising concerns about the overall safety of administering powerful toxins, albeit coupled to a targeting molecule, to humans (https://investor.magentatx.com/news-releases/news-release-details/magenta-therapeutics-voluntarily-pauses-mgta-117-phase-12-dose).

The egress of resident HSPCs from the BM during drug-induced mobilization might generate a window of opportunity for engraftment of gene-corrected cells and has been exploited in preclinical models of conditioning regimens.[265] Transient overexpression of CXCR4 in transplanted gene-edited HSPCs provided an engraftment advantage to infused cells, promoting efficient HSPC exchange in BM niches and stable long-term chimerism in mice.[265–267]

HSPC-GT might gain significant benefit from alternative conditioning regimens, representing a unique clinical context in which new conditioning strategies can be tested and successfully developed, given the autologous source of the infused cell product and that a mixed chimerism might be sufficient for therapeutic benefit in many diseases amenable to GT.

CONCLUSIONS AND FUTURE PERSPECTIVES

Nearly thirty years of research and development of gene transfer vectors have provided the groundwork for successful clinical development and consolidation of HSPC-GT for a growing number of inherited hematological and metabolic diseases. The recent advent of GE bears the promise of even more precise and potentially safer genetic engineering. These advances are the outcome of a multidisciplinary cross-fertilization process from different basic and clinical research areas, including stem cell biology, biochemistry, omics sciences, bioinformatics, and hematology. New concepts and technologies are looming on the horizon with the potential to empower the design of next-generation HSPC-GT, such as nanotechnologies for stealth and targeted delivery,[268] RNA-based instruction of gain-of-function

Table 1. Choosing the proper tool: summary of main therapeutic modalities, upsides and downsides, constraints, and current stage of development for HSPC-GT

Platform	Therapeutic modalities	Upsides	Downsides	Constraints	Stage of development
γ-Retroviral vectors	Gene addition for: • Gene replacement • Instructing new function • Inhibiting endogenous gene expression by micro/shRNA	Long-established manufacturing and clinical use	• Insertional genotoxicity aggravated by strong enhancers in vector LTRs • Expression by reconstituted promoter in the vector may not reproduce physiological regulation	Inefficient in long-term HSCs Semi-random integration profile Expression from viral LTRs	Several past clinical trials/market approvals for one product
SIN lentiviral vectors	•	• Highly efficient gene transfer (also in long-term HSCs) • Robust expression at most ISs • Solid clinical track record with long-term safety and efficacy	• Residual insertional genotoxicity alleviated by SIN LTRs and promoter choice • Expression by reconstituted promoter in the vector may not reproduce physiological regulation	Semi-random integration profile	Several past and current clinical trials/market approvals of a growing number of products
Designer nucleases	• Disruption of coding or regulatory sequences (also multiplex) • Suppression of dominant mutations by allele-specific knock-out	• Site-specific activity • Highly efficient disruption (up to biallelic)	• Adverse effects of DNA DSBs on long-term HSCs (p53 response) • gRNA-dependent off-target activity Large genomic rearrangements at the target site(s)	• Heterogeneous repair outcomes • Influence of interindividual genetic variability on the off-target profile • *Immunogenicity of the editor*	Early-stage clinical trials
	• Correction of mutant gene Targeted integration of: • "One-size-fit-all" corrective cDNA • Expression cassette to safe harbor	• Site-specific activity • Compatible with long-range edits • Preserves physiological regulation of the target gene	• Requires delivery of non-viral/viral DNA templates for HDR (AAV6, IDLV, and ssDNA) • Adverse effects of DNA DSBs and DNA templates on long-term HSCs (p53 response) • gRNA-dependent off-target activity • Large genomic rearrangements at the target site(s)	• Requires HSPC cycling • Low efficiency of gene correction in HSCs • Heterogeneous repair outcomes • Influence of interindividual genetic variability on the gRNA off-target profile • *Immunogenicity of the editor*	
Base editors	• Disruption of coding or regulatory sequence • Correction of point mutation (including dominant ones) • Installment of single nucleotide variants (SNVs) providing novel function • Multiplex editing	• Site-specific activity • Low DNA DSB burden • Highly efficient (particularly adenine base editors)	• Bystander editing and residual DSB generation at target site(s) • Residual gRNA-dependent off-target activity and large genomic rearrangements • *gRNA-independent off-target activity on DNA/RNA (deaminase)*	• Limited to single nucleotide transitions • Mutation-specific gRNA must be designed for gene correction • *Influence of interindividual genetic variability on the off-target profile?* • *Immunogenicity of the editor?*	Close to clinical trial (NCT05456880)

(*Continued on next page*)

Table 1. *Continued*

Platform	Therapeutic modalities	Upsides	Downsides	Constraints	Stage of development
Prime editors	• Disruption of coding or regulatory sequence • Correction of point mutations (including dominant ones) • Installment of small sequence variants providing novel function • *Multiplex editing*	• Site-specific activity • Low DNA DSB burden • Enable all types of nucleotide changes and mostly short-range insertions/deletions	• gRNA scaffold incorporation and residual DSB generation at the target site(s) • Residual gRNA-dependent off-target activity and large genomic rearrangements • *gRNA-independent off-target activity on DNA/RNA (reverse transcriptase)?*	• Suboptimal efficiency • Complexity of emerging multi-component variants enabling long-range edits • Mutation-specific gRNA must be designed for gene correction • *Influence of interindividual genetic variability on the off-target profile?* • *Immunogenicity of the editor?*	R&D stage
Epigenetic editors	• Gene knockdown by heritable silencing • Heritable gene (over) expression • *Multiplex editing*	• Site-specific activity • Do not require DNA DSBs or SSBs	• *gRNA-independent off-target activity (e.g., methyl transferase)?*	• *Long-term stability of the epigenetic change?*	R&D stage

Italic descriptions with or without question marks mean that these concepts have not been shown yet in general or in human HSPCs, respectively.

and chemotherapy-free HSCT,[265] three-dimensional cell culture and bioprinting,[204,269] *ex vivo* expanded and pluripotent stem-cell-derived HSCs,[199] artificial intelligence,[270–272] and prospectively groundbreaking single-cell omics.[273]

Long-term molecular follow-ups in a growing number of patients and diseases will establish at clonal level the safety and resilience of hematopoietic grafts established with LV-engineered cells and open the way to broader applications to less severe as well as acquired diseases such as cancer and neuroinflammatory/degenerative diseases, exploiting HSPC engineering for tissue-targeted delivery of biotherapeutics by their progeny.

The expanding toolbox of GE tools will provide new opportunities for designing safer and innovative gene correction strategies and capturing emerging biological knowledge on HSC biology and regulation to serve the purpose of more sophisticated and powerful engineering. The concerns raised for potentially harmful cellular responses and adverse editing outcomes are forcing iterative rounds of deeper platform assessment and further engineering. Next-generation GE tools may avoid generation of any DNA break or sequence mutation, for instance by stably shutting down gene expression by epigenome editing[79,80] or by limiting exposure to free DNA ends by transposases or engineered site-specific recombinases.[92,274] Given the expanding toolbox for genetic engineering, the choice should be based on the fit of each tool to the intended therapeutic strategy, maximizing the predicted safety and efficacy.[275] In Table 1, we summarize for each major family of genetic engineering tools the main therapeutic modalities, upsides and downsides, constraints, and current stage of development.

The development of more efficacious and/or predictably safer HSPC-GT products such as those offered by GE must be stringently validated by long-term clinical monitoring of treated patients and thorough assessment of the occurrence and consequences of unwanted repair outcomes upon GE. While the first HSPC GE clinical trials conducted so far have reported excellent therapeutic benefits and no evidence of cell transformation,[64] it will be important to learn about the hematopoietic graft clonality, off-target editing, and occurrence/persistence of genomic rearrangements. This information will be extremely valuable to accrue knowledge on the actual risks and limitations of HSPC GE in "real life" scenarios, helping to focus development of improved or novel platforms.

Genetic engineering of human HSPCs *in vivo*, rather than *ex vivo*, may represent the ultimate goal of HSPC-GT because it does not require cell harvesting, nor does it expose them to the potential adverse effects of *ex vivo* manipulation, and it might bypass the conditioning requirement. Furthermore, it would reduce the treatment complexity and costs, potentially widely broadening GT access. *In vivo* HSPC-GT, however, is not an easy task, notwithstanding early preclinical proof-of-concepts in mice and NHPs,[276–278] and faces the intrinsic safety challenges related to biodistribution. Reaching a sufficient proportion of resident HSCs to establish a therapeutically effective chimerism with modified cells might be difficult, at least without coupling gene correction to a selection strategy. Bystander modification of off-target cell types inside and outside the BM might increase the probability of generating cells bearing genotoxic hits and trigger adverse reactions such as immune

sensitization to the editing machinery and transgene product. Moreover, *in vivo* HSPC-GT intrinsically bypasses the release specifications of *ex vivo* GT, which are performed on modified cells rather than on the manipulation machinery, thus greatly hampering characterization of the engineered cell product and investigation of potential detrimental consequences. Whenever applicable, *in vivo* HSPC-GT is also expected to be benchmarked against the competing *ex vivo* approach, which is supported by an ever-growing successful track record and the nearly half century of HSCT experience.

Overall, HSPC-GT may qualify as the next transforming medical approach born out of growing knowledge of HSC biology after the successful clinical development of HSCT.

ACKNOWLEDGMENTS

Work in the labs of R.D.M., A.K.R., E.M., G.F., A. Aiuti, and L.N. is supported by grants from Fondazione Telethon, the EU Horizon 2020 Program (UPGRADE to L.N. and X-PAND to R.D.M. and L.N.), the Italian Ministry of Health (GR-2019-12369499 to S.S., GR-2018-12366006 to A.K.R, and E-Rare-3 to L.N.), the Italian Ministry of University and Research (PRIN2017 to L.N.), the European Hematology Association (to S.F., A. Aprile, and R.D.M.), the New York Stem Cell Foundation (to R.D.M.), the European Research Council (ReviveSTEM to R.D.M. and ImmunoStem to A.K.R.), the Lady Tata Memorial Trust (to A.C.), the American Society of Hematology (to A. Aprile), the Louis-Jeantet Foundation through the Jeantet-Collen Prize for Translational Medicine 2019 (to L.N.), and the Else Kröner Fresenius Foundation through the Kröner-Fresenius Prize for Medical Research 2020 (to A. Aiuti). E.V. contributed to this Review as partial fulfillment of her Ph.D. A.C. is an EHA and ASH-TRTH early career hematological scientist. R.D.M. is a New York Stem Cell Foundation Robertson Investigator.

AUTHOR CONTRIBUTIONS

All authors contributed to writing this Review. S.F. and L.N. coordinated the work and integrated all contributions. The other authors are listed in order of seniority in the field.

DECLARATION OF INTERESTS

L.N. is an inventor on patents on LV technology and gene editing filed by the Salk Institute, Cell Genesys, Telethon Foundation, and/or San Raffaele Scientific Institute. S.F., A.C., R.D.M., and A.K.R. are inventors of patents on gene editing, and E.M. is an inventor of patent on clonal tracking, owned and managed by the San Raffaele Scientific Institute and Telethon Foundation. L.N. is a founder, equity owner, consultant, and member of the scientific advisory board of Genenta Science, a biotechnology company aiming at developing cancer gene therapy by tumor-infiltrating monocytes; GeneSpire, a biotechnology startup developing LV-based liver gene transfer and hematopoietic cell gene editing; and Epsilen Bio/Chroma Medicine, a company developing epigenetic editing. A. Aiuti is a PI of clinical trials sponsored by Orchard Therapeutics, which licensed several gene therapy products originally developed at SR-Tiget. A. Aiuti is a member of the EU Committee for Advanced Therapies (CAT), and his views are personal and may not be understood or quoted as being made on behalf of the European Medicines Agency (EMA). All other authors declare no competing interests.

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