Homology-Mediated Gene Editing

Subjects: Biochemistry & Molecular Biology

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Homology-directed gene editing of hematopoietic stem and progenitor cells (HSPCs) is a promising strategy for the treatment of inherited blood disorders, obviating many of the limitations associated with viral vector-mediated gene therapies.

Keywords: gene editing ; hematopoietic stem and progenitor cells ; homology-directed repair ; CRISPR-Cas9 ; AAV6

1. Introduction

The modification or insertion of genes was initially proposed in the early 1970s as a curative approach for inherited disorders[1]. Hematopoietic stem cells (HSCs) are preferred targets for genetic therapies owing to their ability to sustain lifelong hematopoiesis, affording the possibility of durably alleviating a range of conditions. Current gene therapy approaches for inherited blood disorders primarily entail the harvest of hematopoietic stem and progenitor cells (HSPCs) from individuals with an underlying genetic defect and their adoptive transfer after genetic modification ex vivo (Figure 1a). Decades of allogeneic HSPC transplantation performed in the clinic provided a roadmap for therapeutic translation of this novel approach. The avoidance of allo-reactive complications and the reduced complexity of conditioning regimens in autologous transplantation of genetically modified HSPCs provide substantial advantages over allogeneic HSPC transplantation options for these disorders.

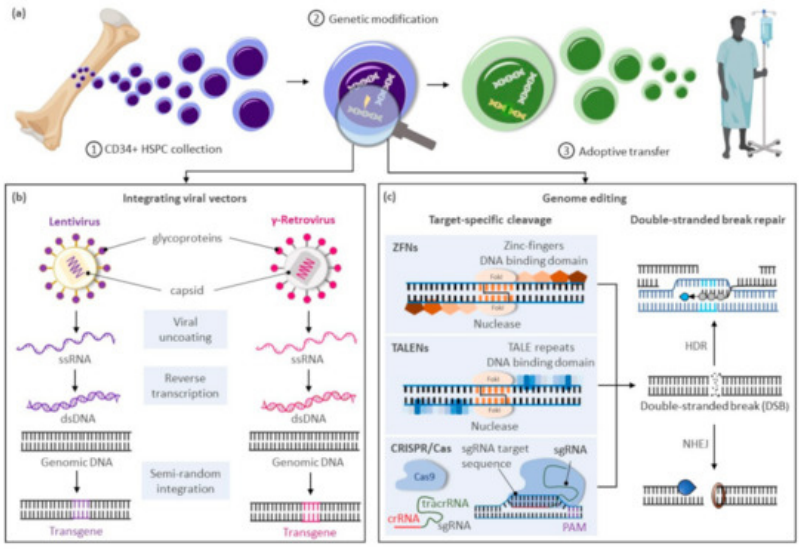

Figure 1. Gene therapy approaches for inherited blood disorders. (**a**) General scheme for gene therapies of inherited blood disorders: (**1**) Isolation of CD34+ hematopoietic stem and progenitor cells (HSPCs) from bone marrow harvests or mobilized peripheral blood cell collections; (**2**) Ex vivo genetic modification of HSPCs; (**3**) Adoptive transfer of genetically corrected cells to the patient generally following a reduced intensity conditioning regimen to enhance engraftment of the treated cells. (**b**) Largely random pattern of transgene integration within the target cellular genome after genetic modification of HSPCs using integrating viral vectors based on lentiviruses or gamma-retroviruses. (**c**) Precise integration of therapeutic genes using genome editing approaches based on zinc-finger nucleases (ZFNs), transcription activator-like effector (TALE) nucleases (TALENS), or the clustered regularly interspaced palindrome repeat (CRISPR)-associated (Cas) platform. Abbreviations: crRNA, CRISPR RNA; dsDNA, double-stranded DNA; DSBs, double-stranded breaks; HDR, homology directed repair; NHEJ, non-homologous end-joining; PAM, protospacer-adjacent motif; sgRNA, single guide RNA; ssRNA, single-stranded RNA; tracrRNA, trans-activating CRISPR RNA.

Clinical trials using gene delivery vectors based on γ-retroviral vectors were initially approved in the 1990s, but only low numbers of corrected cells were detected, and phenotypic correction of the underlying defect was not observed. A refocus on optimizing ex vivo transduction conditions and addition of conditioning regimens to favor engraftment of transduced cells led to the first unequivocal clinical successes in patients with primary immunodeficiencies[2][3][4]. However, the subsequent reporting of malignancies caused by vector-mediated insertional activation of proto-oncogenes in treated patients[5][6][7] encouraged the development of alternative vector designs primarily based on retroviruses of the HIV-1 Lentivirinae subfamily (Figure 1b). Unique constituents of lentiviral vectors facilitate their nuclear translocation within non-dividing HSPCs, further enhancing transduction of these cells. The elimination of portions of the 3′-LTR promoter and enhancer elements in these vectors also provide a key self-inactivating (SIN) safety feature to alleviate concerns on possible recombination with endogenous HIV particles or unintended activation of proto-oncogenes near the genomic site of vector integration. For these SIN vectors, however, the efficiency of transgene expression is highly dependent on the addition of an internal ubiquitous promoter, and reduced or ectopic expression of the therapeutic gene can be limiting in disorders requiring robust or targeted transgene expression for a therapeutic effect.

In recent years, transformative advances have emerged to precisely edit cellular genomes (Figure 1c). Unlike integrating vectors, which can only facilitate gene addition within undefined loci of the cellular genome, novel editing strategies can mediate precise gene correction, gene ablation, and targeted gene addition within cells. Hence, these technologies further address safety concerns associated with integrating vectors, allow more robust and physiologic gene expression by targeted integration of transgenes near endogenous promoters, and extend gene therapies to dominant negative disorders requiring replacement of abnormal gene products rather than simple gene addition. Building on three decades of scientific advances and clinical successes using integrating viral vectors, targeted gene editing in HSPCs is now undergoing similar accelerated pre-clinical and clinical development. In this review, we summarize the current state of targeted gene delivery in HSPCs and examine new strategies developed to improve gene editing efficiency to levels necessary for effective treatment of inherited blood disorders.

2. The Process of Genome Editing

High-efficiency targeted genome editing in mammalian cells generally depends on the initial introduction of a DNA double-stranded break (DSB) at the chosen genomic locus to stimulate cellular DNA repair to yield desired outcomes. As summarized in this section, various cellular nucleases have been engineered to recognize individual target sequences and induce the necessary DSBs and DNA repair response for targeted DNA modification (Figure 1c). Alternative strategies to manipulate cellular genomes that do not rely on double-stranded DNA cleavage, including base editors[8][9][10][11], prime editors[12], and transposases/recombinases [13][14][15][16], were also developed in recent years and have been reviewed elsewhere[17].

2.1. Targeted DNA Double-Stranded Breaks with Engineered DNA Ucleases

Programmable DNA nucleases emerged in the late 1990s and early genome editing studies relied on protein guided synthetic zinc-finger nucleases (ZFNs) (Figure 1c)[18]. ZFNs consist of a non-specific FokI nuclease domain and a finger domain that provides DNA binding specificity. Each amino acid within the finger domain recognizes three DNA base pairs (bp), with several domains required to recognize a 9–18 bp motif. Specific DSBs are made upon dimerization of ZFNs at their FokI domains on opposite strands of the DNA. Zinc-finger nucleases were first shown to successfully edit drosophila DNA in 2002[19] and subsequently in primary human T-cells in 2005[20]. ZFNs have now entered clinical trials[21][22], but their widespread use has been hindered by constraints of the DNA-triplet recognition motif and the specialized expertise required to customize the DNA binding nuclease effector proteins for each genomic target site.

A more versatile transcription activator-like effector (TALE) DNA binding domain from the *Xanthomonas* spp. proteobacteria[23] was subsequently tethered to the same Fok1 endonuclease domain found in ZFNs to create TALE nucleases (TALENs) (Figure 1c)[24]. TALE domains are modular arrays of conserved repeats of 33–35 amino acids in length. Each repeat binds to a single nucleotide within the target sequence with a binding specificity dictated by the repeat-variable di-residue (RVD) at amino acid positions 12 and 13 of the TALE domain[25]. TALENs have been successfully used in pre-clinical models to edit HSPCs at the *CCR5* locus for treatment of HIV[26] and correct the sickle cell mutation in *HBB* with a single-stranded oligonucleotide (ssODN) donor template[27]. While TALENs' RVD-DNA recognition code facilitates the design of binding domains with a broader targeting range than ZFNs, TALEN-based gene editing technologies still entail the complex assembly of nucleases specific to each targeted DNA locus.

The bacterial clustered regularly interspaced palindrome repeat (CRISPR) and the CRISPR-associated (Cas) protein, known as CRISPR/Cas, constitutes a novel class of RNA-guided programmable nucleases with unique simplicity and flexibility for targeted gene therapies (Figure 1c)[28]. Identified as a bacterial adaptive immune system[29], CRISPR

destroys foreign DNA using the Cas endonuclease in a sequence-specific manner. These naturally occurring immune systems have been categorized as either CRISPR-Cas class 1, which requires complexes composed of several effector proteins for cleavage, or class 2, which allows cleavage of nucleic acids with a single effector domain. Due to their simpler requirements, systems based on class 2 have been favored for genome editing. Class 2 is further partitioned into types II (Cas 9), V (Cas 12), and VI (Cas 13). The type II CRISPR/Cas9 system derived from *Streptococcus pyogenes* (SpCas9) is currently the most widely used tool for genome editing in hematopoietic and other cellular sources. Cas9 is guided by a dual-RNA complex consisting of a universal trans-activating CRISPR RNA (tracrRNA) that recruits the Cas9 protein, and a CRISPR RNA (crRNA) with homology to a specific DNA sequence. The system was simplified for genome editing applications by synthetic fusion of both RNAs into a single guide RNA (gRNA). Small chemical groups may also be introduced at the extremities of synthesized gRNA to enhance gene editing, as shown at three therapeutically relevant loci in human HSPCs[30]. The Cas9/gRNA ribonucleoprotein (RNP) complex binds to a cognate proto-spacer adjacent motif (PAM) sequence (i.e., NGG) at the target locus, facilitating heteroduplex formation between the guide RNA sequence and the unwound target DNA strand. Cas9 then undergoes conformational changes, which activate its constituent HNH and RuvC nuclease domains to promote cleavage of both target (i.e., bound to the gRNA) and non-target DNA strands, respectively. The process results in formation of predominantly blunt-ended DSBs upstream of the PAM sequence at the chosen locus.

Several Cas9 variants or alternative Cas proteins have been developed to offset limitations of the CRISPR editing system based on SpCas9. For instance, off-target gene editing at unintended sites may result in deleterious cellular effects. Dual-strand targeting using paired Cas9 nickases derived by mutating the RuvC ($Cas9^{D10A}$) or HNH (H840A) catalytic domains, and two adjacent gRNAs targeting opposing strands of a DNA target[28], can enhance CRIPR/Cas9 accuracy. Similarly, systems based on catalytically inactive Cas9 fused to Fok1 (fCas9), which require recruitment of two Fok1 domains for cleavage[31], can lower the probability of off-target editing. However, design of these systems is more complex, and efficiency is generally lower. Reduced off-target activity was also reported using Cas9 isolated from the alternative bacterial species *Streptococcus thermophilus*[32] and *Francisella novicida* (FnCas9)[33], and from type V CRISPR effector Cas12b derived from *Bacillus hisashii* (BhCas12b)[34]. In HSPCs, the high-fidelity (HiFi) Cas9 mutant improved the on-to-off target ratio when delivered as a purified protein[35], but the potential benefits of other engineered Cas9 variants remain to be determined, as they generally support lower on-target activity[27]. The large cargo size of the CRISPR/SpCas9 system represents another limitation of this technology, precluding packaging within some viral delivery vectors for gene therapy applications. More compact wild-type[36] and mutant[37] Cas9 nucleases from *Staphylococcus aureus* (SaCas9), Cas9 orthologs derived from *Campylobacter jejuni* (CjCas9)[38] and *Neisseria meningitidis* (NmCas9)[39], and type V Cas12e notable for its small size[40] were recently characterized to address this shortcoming. Another disadvantage of the CRISPR/SpCas9 system is the inherent NGG-PAM recognition requirement that limits Cas target site ranges. Several variants have been reported to expand the genome editing armamentarium, such as type V Cas12a nuclease that generally uses orthogonal T-rich PAM sequences[41], NmCas9 that recognizes pyrimidine-rich PAM sequences[39][42], a near PAM-less “SpRY” variant of the prototypical SpCas9[43] and numerous other Cas effectors with altered PAM specificity[44][45].

2.2. Cellular Pathways for Repair of DNA Double-Stranded Breaks

In mammalian cells, DNA DSBs are repaired by classic non-homologous end joining (C-NHEJ), alternative NHEJ (alt-NHEJ, also known as microhomology-mediated end joining, MMEJ), single-strand annealing (SSA), and homology-directed repair (HDR) (Figure 2). The choice of DNA repair pathway after nuclease-mediated DSB formation is influenced by several factors that primarily coalesce on the key role that cell cycle plays in regulating DSB repair[46]. For instance, various phases of the cell cycle will differ in abundance or availability of pathway-specific DNA repair proteins and homologous DNA templates, and the repair mechanism favored may be influenced by the chromatin state of the target cells[47]. In genome editing applications, the DNA end structures induced by distinct programmable nucleases (i.e., blunt ends, 3′ overhangs, or 5′ overhangs) may also trigger distinct cellular pathways for repair of DSBs.

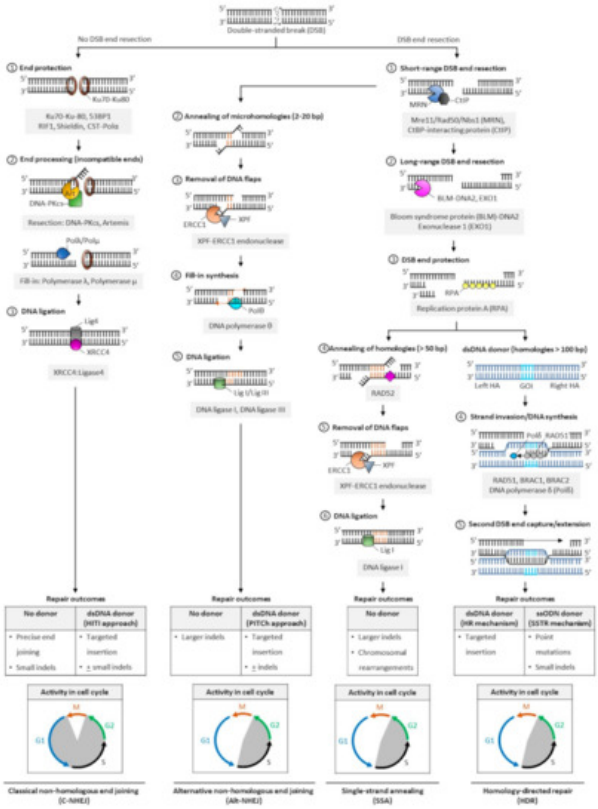

Figure 2. Pathways of DNA double-stranded break repair. Double-stranded breaks (DSBs) are introduced by engineered nucleases at the chosen genomic locus to stimulate endogenous cellular DNA repair mechanisms and promote various repair outcomes. In mammalian cells, DNA DSBs are repaired by classical non-homologous end-joining (C-NHEJ), alternative non-homologous end-joining (alt-NHEJ, also known as MMEJ), single strand annealing (SSA), or homology-directed repair (HDR) pathways. In the absence of donor templates, precise end joining, insertions and deletions (indels), and chromosomal rearrangements may be observed. Addition of a donor DNA template during repair can be used to install and correct point mutations, or knock-in larger DNA sequences. Classic NHEJ does not require template DNA and is the primary repair pathway in cells, whereas alt-NHEJ, SSA, and HDR are known to be active during the S and G2 phases of the cell cycle. Abbreviations: 53BP1: p53-binding protein 1; bp: base pairs; BRAC1: breast cancer type 1; BRAC2: breast cancer type 2; CtBP: C-terminal binding protein; DNA-PKcs: DNA-dependent protein kinase, catalytic subunit; dsDNA: double-stranded DNA; ERCC1: excision repair cross-complementation group 1; GOI: gene of interest; Mre11: meiotic recombination 11; Nbs1: Nijmegen breakage syndrome 1; RAD50/51/52: radiation sensitive 50/51/52; RIF1: Ras relate protein (Rap)-interacting factor 1; CST-Polα: CTC1-STN1-TEN1 (CST)-Polymerase α; HITI: homology-independent targeted insertion; HR: homologous recombination; indels: insertion and deletion; PITCh: precise integration into target chromosome; ssODN: single-strand oligodeoxynucleotide; SSTR: single strand template repair; XPF: xeroderma pigmentosum complementation group F; XRCC4: X-ray repair cross completing protein 4.

The classic form of NHEJ is operational throughout the cell cycle, except mitosis, and quiescent HSPCs largely rely on this mechanism to repair DSB lesions[48][49][50]. Unlike other DSB repair pathways that require DNA end resection at the break site to expose the homology required for repair, broken DNA ends containing no or limited sequence homology (0–4 bp) are ligated in C-NHEJ, and resection is thus not required. Both ends of DSBs are protected from extensive resection by high-affinity binding of Ku70/80 heterodimer complexes[51] and other end protection proteins including the DNA damage response TP53 binding protein 1 (53BP1) and its effectors (RAP1-interacting factor 1 [RIF1], CST complex-polymerase-α [CST-Polα] and the shieldin complex). Compatible DNA ends, such as blunt ends generated by Cas9, are often directly ligated by the XRCC4-DNA ligase 4 (XRCC4:LIG4), with the enhancing activity of XRCC4-like factor (XLF) or paralogue of XRCC4/XLF (PAXX). Incompatible ends, such as 5′/3′-overhangs or 3′-recessed DNA ends, require processing by the Artemis-DNA-PKcs nuclease complex to trim non-complementary end structures, or by Pol μ, Pol λ, and Tdt polymerases to add complementary nucleotides to favor XRCC4:LIG4-mediated ligation. In the absence of donor DNA, the original DNA sequence is generally restored, but limited sequence alterations (e.g., small indels) may also occur at the repair junctions, resulting in silent changes or frameshift mutations leading to target gene inactivation. When a donor sequence is added in vivo along with CRISPR/Cas and gRNA constituents, C-NHEJ can also mediate targeted integration at sites of Cas9-induced DSBs; however, a small percentage of stably integrated sequences may occur in the reverse (undesired) orientation (Figure 2).

The other DSB repair pathways (alt-NHEJ, SSA, and HDR) are known to be active during the S and G2 phases of the cell cycle. They share a first 5′-to-3′ end short-range resection step catalyzed by the Mre11/Rad50/Nbs1 (MRN) endonuclease complex in conjunction with the CtBP-interacting protein (CtIP). This step requires cyclin dependent kinases 1 and 2 (CDK1/2) to phosphorylate and activate CtIP, and is thus limited to the active phases of the cell cycle[52][53]. Recruitment of CtIP to MRN facilitates the removal of Ku70-Ku80 proteins from DSB ends and promotes the dephosphorylation of 53BP1, which in turn inhibits repair by C-NHEJ[54]. This process initially generates 3′ single-stranded overhangs. When short (2–20 bp, most often 3–8 bp) complimentary base pair microhomologies internal to both broken ends are exposed following resection, the broken ends can be repaired by the alt-NHEJ mechanism, involving the annealing of microhomologies, removal of extraneous heterologous DNA flaps by the XPF-ERCC1 endonuclease, fill-in synthesis of the flanking single-stranded regions by DNA Polθ, and sealing by DNA ligases I and III[55][56][57]. Because heterologous flaps flanking the annealed regions of microhomologies are cleaved and lost during alt-NHEJ repair, this pathway is inherently more mutagenic than the classic form of NHEJ. When a donor DNA is added, this repair mechanism can also be exploited for gene knock-in at targeted genomic loci (Figure 2).

In SSA- and HDR-mediated DSB repair, more extensive resection is required. These pathways are thus considerably slower than classical NHEJ or alt-NHEJ mechanisms. The Bloom syndrome protein (BLM)-DNA2 and exonuclease 1 (EXO1) mediate this process, and the replication protein A (RPA) binds the resultant single stranded DNA with high affinity to protect its integrity[58][59]. In SSA, the extended resection exposes longer (> 50 bp) sequences of homologies that are uniquely annealed in a RAD52-dependent manner. Similar to alt-NHEJ, the non-complementary tails are then removed by the XPF-ERCC1 endonuclease complex, and the remaining nicks are sealed by DNA ligase 1[60]. In a normal cellular context, the SSA repair mechanism results in the obligate deletion of a larger sequence between homologous repeats and may promote chromosomal rearrangements (Figure 2)[60].

In HDR[61], RAD51 recombinase is recruited in an ATP-dependent manner to RPA-coated single-stranded DNA, forming a RAD51-DNA nucleoprotein filament. This process is mediated by BRACA2, which is recruited to DNA DSBs by PALB2 and BRAC1 in humans[62][63][64]. The RAD51 ssDNA filament then locates a homologous DNA template. The template is generally a double-stranded sister chromatid available in late S/G2 phases of the cell cycle[52][65][66], or can be provided exogenously in genome editing applications in the form of a double-stranded donor flanked by homology arms. A homology tract of more than 100 bp is typically required as a template to initiate repair by homologous recombination. When complementary ssODN are used as DNA donors, DSBs are processed by a distinct mechanism, the single-strand template repair (SSTR) pathway, which is independent of RAD51 but requires an operative Fanconi anemia (FA) pathway and at least two RAD51 paralogs (RAD51C and XRCC3)[67]. In HDR, the ssDNA filament then invades the homologous region to form a displacement (D)-loop where the template DNA is copied by DNA polymerase δ (Pol δ). The second DSB end is eventually captured by the invading strand, forming a DNA intermediate with two Holliday junctions. This structure undergoes gap repair DNA filling and ligation, and is ultimately resolved at both Holliday junctions in a non-crossover or crossover mode. In some cases, repair can occur by synthesis dependent strand annealing (SDSA), in which the newly replicated DNA dissociates from the template without the formation of a Holliday junction, or by break-induced replication (BIR), when the second DSB end is absent or cannot be found; however, the role of these pathways in genome editing has not been defined (Figure 2). Owing to the obligate use of a donor template sequence, HDR is considered error-free and is generally the preferred pathway for genome editing. Site- and orientation-specific integration at a chosen locus, either upstream of an endogenous promoter or within a safe harbor locus, is a commonly desired repair outcome for therapeutic applications. However, the low frequency of HDR in primary cells, especially long-term repopulating HSCs, remains a challenge to achieving high rates of targeted gene insertion by HDR[48][68].

2.3. Cellular Delivery of Gene Editing Tools

Safe and effective cellular delivery of engineered nucleases, gRNAs, and template sequences constitutes a key step in the process of gene editing. For ex vivo genome editing, approaches for the delivery of the required constituents within target cells can be broadly classified into viral vectors, electroporation, and cell-penetrating peptides[69]. In primary cells, including HSPCs, nucleases and the associated gRNAs are most effectively delivered by electroporation of mRNA molecules or as an RNP complex between gRNAs and the nuclease (e.g., Cas9) protein. Unlike transfection of DNA plasmid molecules, this approach results in limited cytotoxicity to HSPCs. In addition, the short half-life of the complex temporally limits the nuclease activity and the likelihood of genome editing at off-target loci[70].

Donor template delivery has also been a significant challenge for gene editing of HSPCs, as electroporation of dsDNA is highly toxic. Several alternative delivery platforms have been successfully been used, including ssODNs co-delivered with Cas9 [27][71][72][73]. For larger gene insertions, viral vectors are very effective, including integrase deficient lentivirus (IDLV)

[74][75][76] and adeno associated virus serotype 6 (AAV6)[77]. There are several caveats, however, to the delivery of donor templates using virus-based systems, including DNA packaging capacity, which is limited by the viral capsid size, and off-target integration of viral genes[78].

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