CELL & GENE THERAPY INSIGHTS

THE LATEST DEVELOPMENTS IN VIRAL & NON-VIRAL VECTOR MANUFACTURING

SPOTLIGHT

EDITORIAL

Lentiviral vectors in hematopoietic stem cell therapies: mainstay technology, or simply a bridge to gene editing?

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"...the documented clinical success of lentiviral therapies surely means that they will remain an important tool in genomic medicine..."

Hematopoietic stem cells (HSCs) are quiescent progenitors to a range of blood and immune cell lineages with the propensity to repopulate these cellular niches. This makes them attractive as a cell-based therapy for lysosomal storage disorders [1,2], hemoglobinopathies [3] and primary immune deficiencies [4,5]. Indeed, allogenic identical HLA-matched HSC engraftment has been a mainstay therapeutic approach in the field since the 1960s. However, such cases of HLA matches are rare and finding suitably matched donor's remains challenging. Furthermore, in many cases of allogenic HSC transplantation, acute graft versus host disease (GVHD) remains a significant concern. This has fuelled the shift towards use of autologous HSC engraftment, in which the patient's own cells have functional gene expression restored *ex vivo* before reintroduction.

Lentiviral (LV) vectors based on human immunodeficiency virus

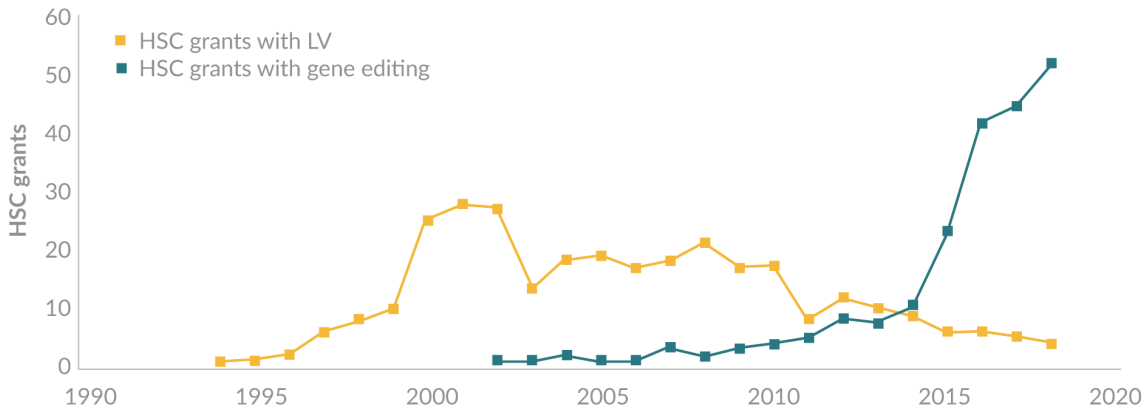
Type-I (HIV-1) have held long-term value in regenerative medicine, due to their ability to permanently integrate therapeutic genes into dividing and non-dividing cells. LV-mediated HSC therapies have been applied successfully in the clinic for a range of disease indications [6–9], with genomic integration patterns showing to be relatively safe, with minimal insertions near proto-oncogenes and clonal expansions [10,11]. However, although further engineering of LV vector technology [12] and alternative retroviral vectors [13,14] may help maintain their future development in HSC therapies, their scientific novelty is rapidly diminishing. As such, there is a growing trend towards replacing LV vectors with gene editing technologies in preclinical development, with the long-term picture suggesting that LV will be almost wiped-out in years to come (Figure 1). This review will comment on the technological advantages and limitations of gene editing therapies, with respect to how their burgeoning popularity could impact on the future use of LV vectors in autologous HSC therapeutics.

CLINICAL TRANSLATION OF GENE EDITING IN HSC THERAPIES: RAPID PROGRESS, BUT SIGNIFICANT CHALLENGES REMAIN

Conventional gene editing utilizes designer endonucleases that can be targeted to and directly cleave the genome at defined sequences, of which there are four main classes: Meganucleases (MGN) [15,16], Zinc Finger Nuclease (ZFN) [17], Transcriptional Activator Like effector Nucleases (TALENs) [18] and the most current Clustered Regularly Interspaced Palindromic Repeat/ Cas9 (CRISPR/Cas9) (for detailed review see [19]). Through the exploitation of diverse cellular DNA repair pathways, the double stranded break (DSB) induced by these reagents can then be resolved in a manner that directly modifies the patient's genome and by extension the disease-causing mutation. Two canonical cellular DNA damage pathways that can be exploited to enable gene correction, addition and deletion or disruption include: non-homologous end joining (NHEJ)

FIGURE 1

The emergence of gene editing technologies in HSC therapies.



Grant statistics obtained from Grantome.com show that since the turn of the decade, LV vectors have steadily been replaced by gene editing technologies in preclinical development of HSC therapies.

DOI: 10.18609/cgti.2018.092

characterized by direct ligation of two DNA termini with intervening small sequence insertions and deletions (InDels), and homology-directed repair (HDR) in which precise modification of the DNA can be achieved through the introduction of a DSB with an exogenous repair template [20].

Gene editing of autologous HSCs has rapidly progressed over the last decade, with ZFN-edited HSCs now in clinical trial for treatment of HIV and sickle cell disease [21,22]. CRISPR/Cas9 has steadily become a dominant tool in preclinical development of HSC therapies, but its potential for broad application in the clinic has recently faced significant questions.

A recent preprint has highlighted a potentially important limitation of CRISPR/Cas9 therapies, in which anti-Cas9 were detected in healthy human sera [23]. Antibodies against *Staphylococcus aureus* Cas9 and *Streptococcus pyogenes* Cas9 were detected in 79% and 65% of samples, respectively. Additionally, anti-Cas9 T cell responses were detected at rates of 46% to saCas9. This undoubtedly has important implications for systemic expression of Cas9 for body-wide, *in vivo* gene editing, although its relevance to *ex vivo* HSC therapies is of less concern if Cas9 expression can be restricted to transient duration prior to transplantation. Thus, the delivery system of gene-editing reagents and the resultant expression profile remain continual and ongoing considerations for developing therapeutic strategies. As such, Cas9 has been delivered to HSCs as plasmid (DNA) [24], mRNA [25] and Ribonucleotide protein complexes [26]. The shift towards the use of ribonucleotide proteins (RNPs) in the field is occurring in the pursuit of 'hit and run' gene editing, in which the expression of the Cas9 protein is high albeit transient.

> "Translational gene editing also faces complex logistical challenges, given that an entire preclinical development pathway will theoretically be required for each individual."

Additionally, recent data has highlighted that deleterious genotoxic events could occur in some gene editing scenarios, where large deletions and chromosomal rearrangements followed DSB repair, leading to chromosomal instability [27]. Damage associated with DSBs could be minimized through stringent design and validation of single-guide RNAs (sgRNAs), to minimize 'off-target' effects, which can occur at unintended genomic sites containing up to six mismatches to the sgRNA sequence [28]. Multiple strategies are being employed to address this, such as empirical research to aid and inform in silico guide design [29–31] and a multitude of genome wide off-target assessment methods such as guide-seq [28], digenome-seq [32] and circle-seq [33] to permit judicious sgRNA selection. Protein engineering of the Cas9 endonuclease has also been undertaken and has resulted in numerous variants, including nickases [34], Cas9-fokI fusions [35] and high-fidelity variants with 'neutralizing base' substitutions at the RNA-DNA interface [36,37].

Optimizing the delivery and specificity of current gene editing

technologies are of crucial importance to efficient therapeutic gene editing, but a further technical challenge lies in the scalability of this approach and biasing the cellular DNA repair pathway to the appropriate DSB resolution pathway. Many therapeutic HSC strategies are reliant upon HDR to achieve genetic correction, which is a particularly inefficient process in quiescent HSCs, due to HDR and NHEJ working antagonistically throughout the cell cycle. A multitude of approaches have sought to bias cellular DNA correction to a HDR repair profile, including small molecule inhibitors that target and supress range of protein mediators throughout the NHEJ DNA repair pathway (reviewed in [38]), cellular synchronisation to late G2/S phases [39] and overexpression of HDR protein mediators [40,41]. However, the long-term effects of these pharmacological treatments on the propensity of HSC to undergo self-renewal and differentiation remains to be determined. In addition, integration methods independent of conventional HDR are subject of on-going investigation and studies including homology independent transgene integration (HITI) [42], microhomology end joining (MMEJ) [43] and Rad51 independent single strand annealing (SSA) integration [44].

An alternative method being explored for HSC gene editing is 'base editing', which theoretically would not be restricted to particular phases of the cell cycle. Base editing is mediated through delivery of Cas9 orthologues fused to enzymes that catalyze single nucleotide mutations. The first generation of these utilized APOBEC-mediated cytidine deamination to affect a C•G to T•A transition [45] and was later expanded to adenine deaminases with the ability to convert A•T to G•C base pairs [46]. The main advantage of the technology is that resulting genomic lesions are primarily composed of single-stranded 'nicks' and thus less deleterious to the genome than full DSBs. Notably, low level DSB events do occur with this system, inspiring further optimisation of base editors by limiting DSB formation with additional modifications [47] and directed evolution strategies [46]. Furthermore, fusion of these base editing enzymes to other Cas9 orthologues has served to further expand the scope of potential sites that could be edited and improve amenable delivery strategies [48].

As outlined previously, LV technology has been successfully applied in scenarios suited to unregulated expression of the therapeutic transgene. However, as HSC gene therapy expands though an increasing range of disease indications, it may be necessary to incorporate more sophisticated transcriptional control over transgenes that may not be appropriate to constitutive expression. Although this has been successfully applied to treatment of beta-thalassemia, the use of complex non-coding sequences in some vectors has led to aberrant transcriptional effects in clinical trials [49]. Harnessing of endogenous gene regulation is likely to present a favorable approach for the treatment of the majority of disease indications. But in some cases, supraphysiological expression of the transgene product is an important feature of LV-HSC therapies, in order to provide adequate cross-correction of the

DOI: 10.18609/cgti.2018.092

required tissues [50–53]. In these cases, endogenous gene expression levels may not be sufficient, meaning that conventional gene editing would be unfavorable.

Translational gene editing also faces complex logistical challenges, given that an entire preclinical development pathway will theoretically be required for each individual genomic target. Additionally, as we have described in previous sections, gene editing therapies have very precise requirements in their design, which currently restricts the application of a single technology to the treatment of all patient genotypes for a given disease indication. Indeed, CRISPR/Cas9 methodologies are far more diverse than LV designs, which could permit treatment of a broad range of genotypes. But this creates a potential headache from a commercial perspective, as a single company would likely need to acquire access to multiple patents in order to treat the majority of patients within a single disease area. Either that, or firms would need to be built around a single CRISPR/Cas9 platform technology and spread its application across a broader range of diseases. Either way, it is likely that commercial gene editing therapies will follow a different path to those incorporating LV.

CONCLUDING REMARKS

With LV vectors providing the ability to treat the vast majority of patient genotypes, along with growing evidence for clinical safety and efficacy, it is likely that their use will hold long-term value in *ex vivo* regenerative medicine. But specific diseases and mutations might necessitate use of gene editing in scenarios requiring strict regulatory control over the therapeutic gene. The most likely scenario is that both LV vectors and gene editing technologies will acquire niche applications in the clinic, with their use dictated by the characteristics of a given disease and the common genotypes across patient populations. The shift in popularity towards gene editing in preclinical studies can of course be partly explained by the diminishing novelty of LV technology, although the documented clinical success of LV therapies surely means that they will remain an important tool in genomic medicine for years to come.

FINANCIAL & COMPETING INTERESTS DISCLOSURE

The author has no relevant financial involvement with an organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock options or ownership, expert testimony, grants or patents received or pending, or royalties. No writing assistance was utilized in the production of this manuscript.

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DOI: 10.18609/cgti.2018.092

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DOI: 10.18609/cgti.2018.092