Combinatorial testing of viral vector and CRISPR systems for precision genome editing

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Chapter 2

"Soft" genome editing using CRISPR nickases as a potential source of safer cell products

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Cell & Gene Therapy Insights 9:1201-1210 (2023)

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Abstract

The integration of the gene and cell therapy fields through the application of genome editing principles permits generating *ex vivo* transplantable grafts from stem cells or from their differentiated progenies (*e.g.*, T and NK cells) with novel genetically-engineered function(s). As such, these technologies are offering new therapeutic avenues to previously intractable inherited and acquired disorders (*e.g.*, malignant and infectious diseases). In this article, we discuss the main characteristics, advantages and limitations of genome editing involving the targeted chromosomal insertion of transgenes upon site-specific double-stranded DNA break (DSB) formation by programmable nucleases, namely, RNA-programmable CRISPR nucleases. Subsequently, building on this information and recent findings, we put forward the view that targeted transgene insertion strategies based on CRISPR nickases, as opposed to nucleases, address important limitations of conventional DSB-dependent genome editing approaches. In particular, the cytotoxicity and high genotoxicity resulting from DSBs especially in cell types highly sensitive to DNA damage, including pluripotent and hematopoietic stem cells.

Background

Genome editing or genome engineering is a fast-evolving field with growing impact on basic science, biotechnology, and medicine [1]. Particularly versatile genome editing strategies consist of inserting exogenous donor DNA constructs into specific genomic loci (knock-in) subjected to double-stranded DNA breaks (DSBs) made by engineered nucleases derived from class 2 type II CRISPR systems consisting of single guide RNA (gRNA) and Cas9 ribonucleoprotein complexes (CRISPR-Cas9 nucleases) [2]. This versatility stems from the robust activity and straightforward designing of these RNA-programmable nucleases and the amenability of gene knock-in strategies to genomic modifications spanning entire transgenes, including those encoding chimeric antigen receptors (CARs) and T-cell receptors (TCRs) alone or together with auxiliary factors, such as positive-selection markers and safety

genetic switches [3,4]. Indeed, notwithstanding the growing mining for and adaption of CRISPR and CRISPR-like systems for genome editing purposes, engineered CRISPR-Cas9 nucleases based on the prototypic *Streptococcus pyogenes* CRISPR system and their molecularly evolved or structurally-guided designed variants (*e.g.*, high-specificity and targeting range-expanded variants), remain leading tools for a wide variety of genome engineering applications [5,6].

Main attributes of CRISPR nuclease-assisted genome editing

Chromosomal gene knock-in procedures often entail the delivery of CRISPR-Cas9 nucleases together with donor DNA constructs designed as substrates for either homology-independent or homology-dependent repair (HDR) pathways [7]. Generally, HDR-mediated transgene knock-ins are more precise than those resulting from homology-independent processes in that they are naturally inserted at the chromosomal target site in a predefined orientation and present neither multiple-copy insertions nor imprecise 'foot-prints' at the junctions between genomic and exogenous DNA sequences [8,9]. Importantly, as HDR takes place during the late G2 and S phases of the cell cycle, therapeutically relevant dividing cell types, such as induced pluripotent stem cells (iPSCs), natural killer (NK) cells and T lymphocytes, are amenable to precise HDR-mediated genome editing. For instance, in what valuable target cells is concerned, genetically engineered CAR-T cells, serving as personalized 'living drugs', are yielding impressive results in terms of treating CD19-positive hematological malignancies [3,10]. This is so despite their high costs that stem in part from the difficulties in generating the large amounts of the respective engineered cell products. Since 2017, a growing number of these CAR-T cell products have in fact started to be approved by the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) [11]. Building on the resulting CD19-targeted cancer therapy datasets, over 500 CAR-T cell therapies directed at different antigens in liquid and solid tumors are currently undergoing clinical testing worldwide [11,12]. Significantly, CAR-NK cells are also entering the

adoptive immunotherapy field as a potential alternative to CAR-T cells owing to their intrinsic tumor-cell killing activity and fewer adverse effects in patients [13]. Yet, regardless of the target cell type, in what the genetic modification procedures are concerned, the adoptive immunotherapy field is moving from randomly integrating retroviral vector and DNA transposon systems to targeted transgene insertion approaches using programmable nucleases [3,10]. In contrast to unpredictable *CAR* or *TCR* donor construct integration, programmable nuclease-assisted genome editing assures stable and homogeneous transgene expression while minimizing insertional mutagenesis risks inherent to randomly integrating vehicles. In fact, in contrast to random, targeted *TCR* transgene insertion leads to predictable T-cell function *in vivo* [14]. In this context, genomic loci generically dubbed 'safe harbor' are particularly appealing endogenous landing pads for *CAR* and *TCR* transgenes as insertions at these sites minimize the chances for gene silencing or variegated transgene expression while preserving the endogenous transcriptome of engineered cells [15,16].

Main limitations of CRISPR nuclease-assisted genome editing

As aforementioned, programmable nucleases and HDR-tailored donor DNA constructs yield precise gene knock-ins. However, a major limitation regarding the use of programmable nucleases is the fact that, in mammalian cells, DSBs are prevalently repaired via mutagenic non-homologous end joining (NHEJ) or microhomology-mediated end joining (MMEJ) processes instead of accurate HDR [17,18]. Moreover, in contrast to HDR, end-joining processes take place throughout the cell cycle. As a result, amongst cells exposed to donor constructs and programmable nucleases, the vast majority contains one or both target alleles disrupted by NHEJ- or MMEJ-derived small insertions and deletions (indels). This mutagenic burden, in the form of indel 'footprints', can lead to target protein imbalances and cell fitness losses [19]. In addition, on-target DSB formation can also yield translocations and gross chromosomal rearrangements [19–22]. Recent studies have further uncovered that on-target DSBs are capable of triggering extensive

chromosome fragmentation followed by haphazard reassembly (chromothripsis) [23,24] and the partial or entire loss of chromosomes (aneuploidy) [25]. Notably, the chromothripsis and aneuploidy phenomena were readily detected in T cells and hematopoietic progenitor cells subjected to CRISPR-Cas9 nuclease reagents used in clinical trials [23,25]. Notwithstanding these phenomena, recent findings are more reassuring in that, contingent upon gRNA target site selection, chromosomal losses in particular can be substantially minimized by inducing DSB formation before, as opposed to after, the activation/stimulation of the primary T-cell populations [26].

Finally, on-target DSBs trigger P53-dependent cell cycle arrest and apoptosis which limits the efficacy of HDR-mediated genome editing in regular P53-positive cells [27,28], and creates selective pressure for the emergence of mutations associated with tumorigenesis. Related to the latter matter, during sub-culturing, pluripotent stem cells can acquire 'spontaneous' tumor-associated P53 mutations in a recurrent fashion [29] which, by virtue of being more resistant to DSBs, are in principle more prone to expansion than their wild-type counterparts once exposed to programmable nucleases. Indeed, CRISPR-Cas9 nuclease activation of certain signaling pathways can lead to the selection of cells with potentially harmful loss-of-function or dominant-negative mutations in the tumor-suppressor P53 transcription factor or gain-of-function mutations in the KRAS oncoprotein [27,30]. Furthermore, recent mouse modelling experiments indicate that p53 mutant cells, rather than proceeding to malignancy via a haphazard route, are instead subjected to an unexpectedly more deterministic set of genetic instability events [31]. Together, these cytotoxic and genotoxic effects raise tangible concerns on the use of programmable nucleases for the optimal generation of autologous genetically-corrected cell products.

Rationale for "soft" genome editing based on CRISPR nickases

Although emerging high-specificity programmable nucleases can greatly minimize off-target DNA cleavage, *e.g*., eSpCas9(1.1) [32] and Cas9-HF1 [33], they are inherently incapable of eliminating the potentially deleterious effects resulting from on-target DSB formation. Therefore, the substantial genotoxicity and cytotoxicity profiles associated with conventional nuclease-assisted genome editing create a pressing need for the development of alternative genetic engineering systems that reliably generate safer and functionally robust cell products. Indeed, DSB-dependent genome editing is expected to be particularly risky in the context to cell therapies based on the transplantation of populations of genetically engineered pluripotent stem cells, T lymphocytes and NK cells. The reasons are twofold. Firstly, in the context of extensive *ex vivo* cell amplification protocols underpinning the generation of these cell transplantation products, DSB-derived mutations and/or chromosomal rearrangements can cooperate in cell transformation and clonal expansion. Secondly, in instances where targeting multiple genes is needed for achieving a robust anti-tumor effect, *e.g*., via combinatorial exogenous *CAR* transgene knock-ins and endogenous *TCR* or programmed cell death protein 1 (*PD1*) gene knockouts, simultaneous induction of the attendant DSBs at different genomic positions is expected to exacerbate the levels of undesirable genome editing by-products in the form of translocations and chromosomal rearrangements. In this context, investigations exploring alternative HDR-mediated gene knock-in approaches that rely on sequence- and strand-specific nucleases ('nickases') are valuable in that the resulting single-stranded DNA breaks (SSBs), or nicks, are substrates for neither NHEJ nor MMEJ. As a corollary, the balance between precise HDR to undesired end-joining events are dramatically biased towards the former. Moreover, although genomic SSBs are, *per se*, poor HDR stimuli, earlier experiments from our laboratory using the native adeno-associated virus Rep68/78 nickases demonstrated that concomitant SSB formation at acceptor sequences and donor DNA constructs fosters HDR-mediated gene knock-in at an

endogenous human locus, namely, the prototypic safe harbour locus *AAVS1* [34]. The application of this generic *in trans* paired nicking (ITPN) principle was subsequently expanded to other genomic sequences through the use of more versatile RNA-programmable CRISPR-Cas9 nickases [35,36] that are simply obtained through site-directed mutagenesis of one of the two nucleases domains of the parental Cas9 protein (*i.e.*, HNH or RuvC) (**Figure 1**) [37]. Indeed, by stimulating otherwise inefficient SSB-dependent HDR, ITPN approaches based on the delivery of nicking CRISPR-Cas9 complexes and matched nickase-susceptible HDR donor constructs, are valuable for seamless and scarless chromosomal editing, including at multiple-copy or essential genomic tracts [19,38]. Additional examples regarding the application of ITPN methodologies in various mammalian cell types, *e.g.*, iPSCs, keratinocytes and organoids featuring normal or cancer traits, encompass: (i) repairing or installing predefined gene mutations [35,38-41], (ii) maximizing the integrity of unmodified alleles during allele-specific gene editing [42,43], and (iii) streamlining one-step biallelic gene editing or one-step multiplexing gene knock-in or tagging [35,44,45]. It is equally worth mentioning that, in contrast to regular and high-specificity CRISPR-Cas9 nucleases, CRISPR-Cas9 nickases constitute poor P53-dependent signalling triggers in human cells, including in DNA damage-sensitive iPSCs [38,40]. Hence, it is expected that the aforementioned growing mining for CRISPR systems buried in large genomic and metagenomic databases, will start unearthing enzymes that, via either their intrinsic or engineered nicking activities, enlarge the toolset for DSB-free genome editing. Examples include

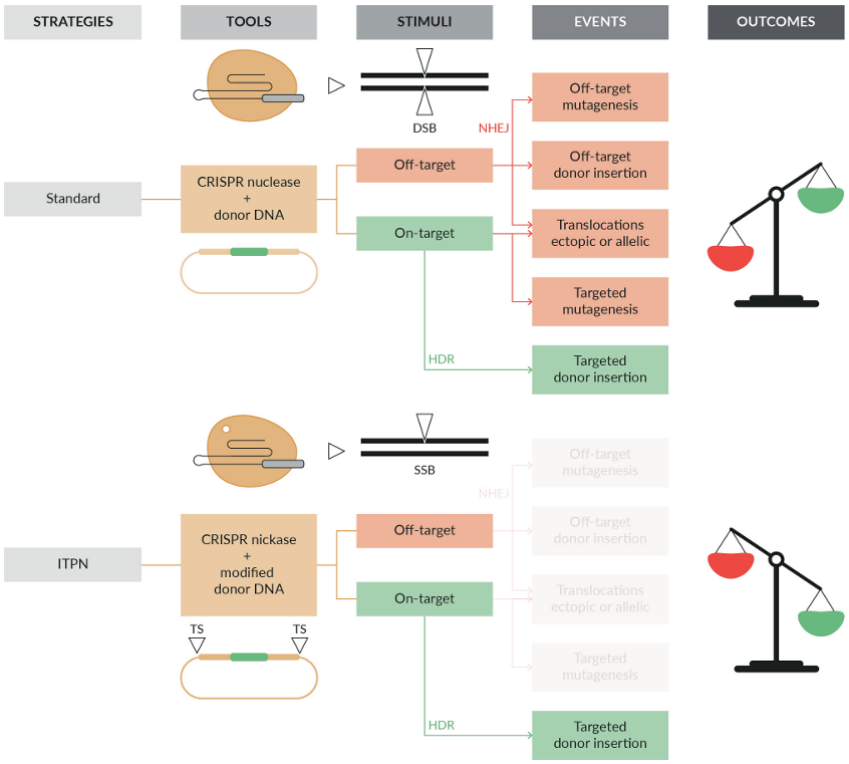

Figure1. Standard versus *in trans* paired nicking genome editing. The relative weights of desired and undesired genome editing outcomes derived from, respectively, homology-directed repair (HDR) and imprecise events caused by competing end-joining DNA repair pathways, *e.g.*, non-homologous end joining (NHEJ), are illustrated. DSB and SSB, double-stranded and single-stranded chromosomal breaks, respectively; 'nickases', sequence- and strand-specific nucleases. In contrast to standard donor constructs, modified donor constructs have nickase-susceptible target sites (TS) framing their targeting modules consisting of exogenous DNA (green bar) flanked by sequences homologous to the genomic target region ('homology arms').

the HNH-negative IsrB nickase derived from the ancestral CRISPR-like system OMEGA and the RuvC-only CRISPR class 2 type V Cas12i nuclease that nick and preferentially nicks, respectively, double-stranded DNA substrates [46–48]. Moreover, often, newly discovered CRISPR systems also yield genome editing components whose small sizes renders them more fitting for delivery through commonly used adeno-associated viral (AAV) vectors [49].

Finally, another recent 'soft' HDR-mediated genome editing concept that might be particularly suited for the repair of heterozygous or dominant mutations involves allele-specific chromosome nicking for the stimulation of interhomolog recombination (IHR) in somatic cells [50,51]. Through this process of allelic conversion, a pathogenic mutation in one allele can, in principle, be corrected using as donor template the endogenous 'healthy' allele (**Figure 2**). This elegant concept of using CRISPR-Cas9 nickases and endogenous homologous chromosomal DNA as repairing templates has been demonstrated in *Drosophila* models [51] and human cell lines [50,52]. Regarding the application of such exogenous donor DNA-free genome editing principles in human cells, recent investigations argue for multiplexing approaches in which primary allelic-specific gRNAs act in concert with secondary gRNAs to direct *in trans* paired nicking of homologous chromosomes and ensuing allelic conversion via IHR (**Figure 2**) [52]. Further research will be instrumental to advance CRISPR-Cas9 nickase-induced IHR from enticing proof-of-concept studies in cell lines to its application in human stem and progenitor cells.

Translation Insight & Outlook

There is a pressing need for investigating and validating alternative DSB-free and precise genome editing tools and strategies in various stem and progenitor cell types, *e.g.*, *bona fide* T and NK cells as well as precursor iPSCs from which different effector cells can be differentiated, including immunotherapeutic T and NK cell candidates. Genome engineering strategies covering targeted and precise chromosomal incorporation of genetic payloads with varying sizes will become ever-more relevant. In this regard, CRISPR nickases *per se* and fused to reverse transcriptases offer a complementary

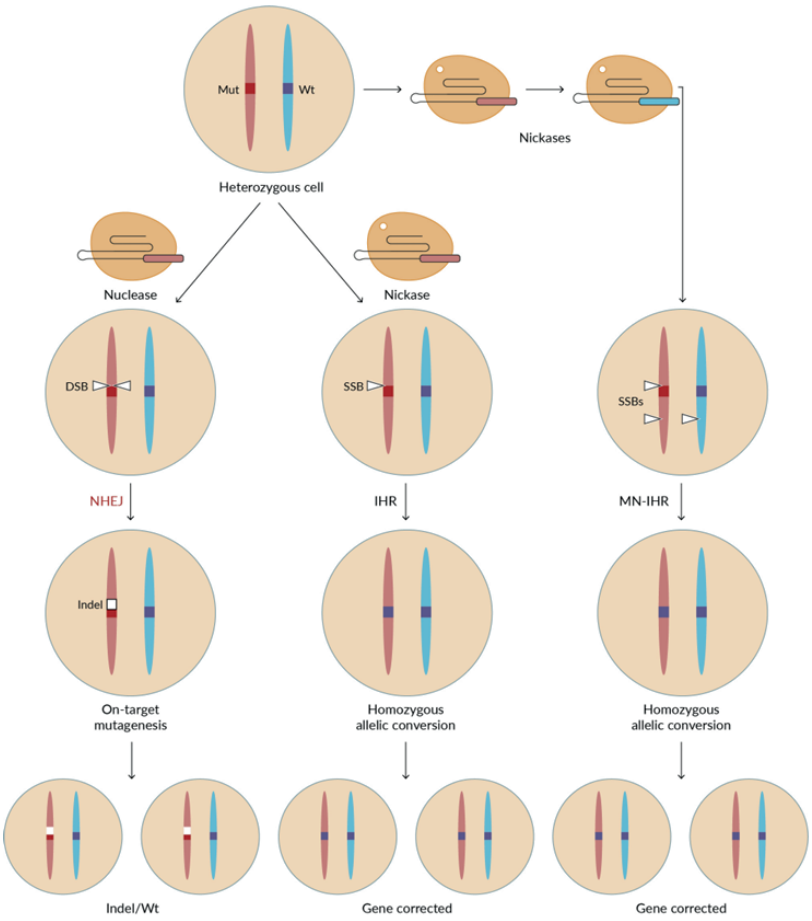

Figure2. Gene correction via interhomolog recombination between heterozygous allelic sequences. Interhomolog recombination (IHR) characteristic of meiosis in germ cells can be fostered in somatic cells subjected to allele-specific double-stranded DNA breaks (DSB), yet the major products are on-target mutagenesis in the form of NHEJ-derived small insertions and deletions (indels). In contrast, allele-specific single-stranded DNA breaks (SSB) can equally foster IHR in somatic cells especially when using multiplexing CRISPR-Cas9 nickases for *in trans* multiple nicking IHR (MN-IHR). In somatic cells with heterozygous mutations or compound heterozygous mutations (not shown) underlying genetic disorders, CRISPR-Cas9 nickase-induced IHR offers the prospect for new genetic therapy interventions via wild-type allele-templated gene repair.

toolbox for 'soft' genome editing involving HDR and prime editing, respectively. Contrary to HDR, prime editing does not require the transfer of donor DNA substrates and allows for genomic insertion of up to ~44-bp of

foreign DNA despite the need for substantial optimization of extended prime-editing gRNAs (pegRNAs) [53]. Moreover, in contrast to HDR-based genome editing, prime editing can take place in post-mitotic cells albeit to lower efficiencies than in cycling cells [54]. Recent prime editing developments include the combinatorial use of dual pegRNAs and site-specific recombinases designed for replacing genomic sequences with up 250-bp of foreign DNA and inserting entire transgenes at prime editor-defined recombinase target sites, respectively [53]. Despite powerful and versatile, such combinatorial strategies require the delivery of large and multicomponent reagents into target cells. An aspect warranting attention when considering multiplexing approaches concerns the importance of introducing balanced amounts of the attendant individual components to maximize the performance and precision of genome editing interventions [55]. In addition, prime editing involving the delivery of dual pegRNAs is not compatible with large edits whereas sequential prime editing and site-specific recombination is not amenable to subtle genomic edits underlying endogenous gene repair due to discontinuous 'footprint' installation in the form of recombinase target sites.

In conclusion, considering the herein discussed findings and matters, one can submit that cell therapy products derived from the use of RNA-programmable nickases as such or with heterologous domains, will offer a complementary set of 'soft' genome engineering options whose safety profiles are potentially higher than those associated with the exposure of cells to programmable nucleases.

Contributions

The named author takes responsibility for the integrity of the work as a whole, and has given his approval for this version to be published.

Acknowledgements

None

Disclosure and potential conflicts of interest

Dr. Gonçalves is a member of the European Reference Network - Neuromuscular Diseases (ERN EURO-NMD).

Funding declaration

Research in the authors' laboratory is supported by the Prinses Beatrix Spierfonds, the Duchenne Parent Project NL, the Dutch Research Council (NWO) - Open Technology Program, and EU Marie Skłodowska-Curie Doctoral Network Actions. Dr. Li holds a PhD fellowship from the China Scholarship Council - Leiden University Joint Scholarship Program

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