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

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Chapter 6

Summary and general discussion

Genome editing (*aliases,* genomic engineering and gene editing) is a fast-paced field with increasing impact on fundamental science, biotechnology, and medicine [1]. With the aid of engineered RNA-guided nucleases (RGNs) derived from clustered regularly interspaced short palindromic repeat (CRISPR) systems initially discovered as prokaryotic antiviral machineries in bacteria and archaea [2], genome editing has become more versatile and customizable with regards to the options for achieving gene knock-out (KO), gene knock-in (KI), targeted DNA replacement and tagging, amongst other chromosomal DNA modification endpoints [1,2]. Despite the increasing discovery and adaption of new CRISPR and CRISPR-like systems for genome editing purposes [3], engineered CRISPR-derived RGNs based on the prototypic *Streptococcus pyogenes* CRISPR-Cas9 system, and their variants created by directed evolution or rational design (*e.g.*, high-specificity and targeting range-expanded variants), remain commonly used reagents for a broad range of genome engineering applications [4]. This stems in large part from their relative robustness in engaging eukaryotic chromatin [5].

Yet, although nuclease-induced double-stranded DNA break (DSB) formation yields robust and cell cycle-independent gene KO endpoints upon the installation of small insertions and deletions (indels) by canonical and alternative non-homologous end joining (NHEJ) pathways, these DNA repair pathways are disruptive to cell genotypes in the context of gene KI procedures based on homology-directed repair (HDR) [6]. Moreover, the possibility for RGN off-target and, more pervasively, on-target activities and ensuing mutagenic effects, which intrinsically stem from NHEJ processes, demands the exploration of more precise and less mutagenic (‘soft’) genome editing strategies. An emerging class of such DSB-independent strategies is reviewed in **Chapter 2** where sequence- and site-specific nucleases ('nickases’) derived from CRISPR systems are exploited for precise HDR-mediated gene KI using tailored donor DNA substrates. Indeed, in contrast to DSBs, single-stranded DNA breaks (SSBs), or nicks, are not substrates for mutagenic NHEJ DNA repair pathways, canonical or otherwise. As corollary, genomic engineering

using nickases is dramatically biased towards precise HDR over imprecise NHEJ events. Of note, although genomic SSBs are *per se* weak HDR stimuli, earlier experiments demonstrated that concomitant SSB formation at acceptor genomic sequences and donor DNA substrates by CRISPR/Cas9 nickases fosters HDR-mediated gene KI [7-9]. The application of this generic *in trans* paired nicking principle yields genomic engineering of stem cells with minimal allelic indels, translocations, and P53-dependent activation of the DNA damage response (DDR) known to trigger apoptosis or, alternatively, halt the cell cycle progression needed for HDR [7,9]. Therefore, as pointed out in **Chapter 2**, cell therapy products derived from using RNA-programmable nickases as such or coupled to heterologous effector domains, like prime editor reverse transcriptases or base editor deaminases, will start to increasingly offer a complementary set of 'soft' genome editing options whose performances and safety profiles are potentially higher than those resulting from exposing cells to programmable nucleases.

In **Chapter 3**, the relevance of DSB-independent genome editing efforts is further elaborated through a commentary to a study by Chai and colleagues [10]. In this study, base editors based on Cas9 nickases fused to deaminase effector domains are tested for the purpose of restoring dystrophin synthesis in *in vitro* and *in vivo* models of Duchenne muscular dystrophy (DMD; MIM: 310200). In particular, the authors identify adenine base editors and cognate guide RNAs that, by installing A·T-to-G·C transitions at splicing motifs, lead to defective *DMD* reading frame repair via exon skipping and subsequent removal of premature stop codons that arose due to out-of-frame deletions. Indeed, by using dual AAV vectors for the *trans*-splicing assembly of complete base editor proteins, the authors demonstrate the rescue of dystrophic traits at the cellular and organismal levels in dystrophin-defective mice upon dual AAV vector administrations. Specifically, base editor constructs linked to N- and C-terminal intein domains were split and packaged in two distinct AAV vectors that, upon target cell co-transductions, led to intein-mediated protein *trans*-splicing and *in situ* assembly of full-

length base editor proteins. This work departs from earlier studies based on dual AAV delivery of RGNs where potentially deleterious outcomes in the form of prevalent end-joining 'capture' of Cas9-encoding AAV genomes at nuclease target sites were detected in various murine tissues, including skeletal muscle [11,12].

It is consensual the view that a key bottleneck regarding the application of genome editing technologies concerns the difficulty in delivering the attendant tools in an effective manner, especially so into regular, non-transformed and primary cells including therapeutically relevant cell types. In this context, physical and chemical transfection methods based on, for instance, electroporation, polyplexes and polycations, permit introducing RGNs and donor DNA constructs into mammalian cells. However, achieving optimal transfection efficiencies without noticeable cytotoxic effects in the aforementioned primary cell types, remains challenging. Moreover, neither electroporation nor transfections of RGNs and/or donor DNA substrates are easily amenable to *in vivo* settings. And, often, these gene-editing tool delivery methods depend on cell type-specific reagents whose compositions may be unknown due to proprietary reasons. Equally of note, electroporation and transfections are further reliant on time-consuming optimizations of cell type-specific parameters whose performances can vary due to subtle experimental conditions, *e.g*. cell-cycle stage profiles of target cell populations. Diversely, viral vector transductions are straightforward to perform and typically offer higher reproducibility independently of the target cell type of choice [13]. These characteristics stem from the fine-tuned mechanisms evolved by the parental viruses for nuclei acid transfer into host cell nuclei.

In **Chapter 4**, inspired by the complementary attributes of two distinct classes of viral gene-free viral vectors, namely, high-capacity adenoviral vectors (AdV) and AAV vectors (*e.g*., large cargo compatibility and source of recombinogenic HDR substrates, respectively), a dual viral vector system is

introduced, characterized, and tested for HDR-mediated gene KI purposes. In this dual viral vector genome-editing platform, RGN constructs are packaged in AdV capsids; whilst donor DNA templates are placed in AAV capsids.

Next to genome-editing tool delivery considerations linked to the ultimate efficiency of gene KI procedures, other parameters to consider regard the specificity and precision of donor DNA insertion. The specificity results from detecting donor DNA sequences at the target site; whilst the precision can be assessed by establishing the precedence of accurate HDR-dependent DNA insertions over imprecise NHEJ-derived events. In this regard, knowledge concerning the relative contributions of distinct AAV donor designs (*i.e.*, conventional versus 'double-cut') and structures (*i.e.*, single-stranded versus double-stranded) to the genome-editing specificity and precision parameters, is scant. Hence, besides achieving remarkably high gene KI efficiencies in transformed, non-transformed and primary cells, the dual viral vector platform studied in **Chapter 4** served as a probe to study the relationship between the aforementioned AAV DNA arrangements and the specificity and precision of gene KI and tagging procedures. This research disclosed that combining single-stranded AAV delivery of HDR donors with high-capacity AdV transfer of RGN constructs leads to precise genome editing in large fractions of target-cell populations. And, it revealed that RGN-induced chromosomal DNA breaks promote productive AAV vector transduction as scored by transgene expression presumably owing to fostering single-stranded to transcriptionally-competent double-stranded DNA conversion. Finally, in **Chapter 4**, it is further disclosed the critical importance of using high-fidelity RGNs for minimizing off-target donor AAV insertions in the form of defective vector genomes known to be packaged in vector particles [14,15].

The aforementioned recombinogenic character of AAV vector genomes, that makes them proficient HDR substrates, also contributes to their promiscuous "capture" at on-target and off-target or random chromosomal DNA breaks

through imprecise NHEJ processes [11,12,16,17]. These end-joining processes can further yield chromosomal insertion of concatemeric and sub-genomic AAV species further compounding the range of HDR-independent bystander events [11,12,17,18]. Finally, possibly due to mimicking DNA lesions or repair intermediates, AAV genomes can impair cell viability through P53-dependent DDR activation whose consequences have been reported to be particularly deleterious in stem cells [19,20]. In conclusion, a growing amount of research indicates that there are distinctive genotoxic and cytotoxic effects linked to chromosomally integrated and episomal AAV DNA forms, respectively, with the build-up of these deleterious effects being strictly proportional to input AAV vector amounts.

Hence, with the aim of tackling the shortcomings associated with AAV-based genome editing, in **Chapter 5**, a marker-free co-selection system [21], dependent on the potent specific inhibitor of the Na^+/K^+ ATPase pump encoded by *ATP1A1*, ouabain, was co-opted and investigated. To this end, AAV donor vectors endowed with a secondary *ATP1A1*-selectable donor module and matched gRNA units were assembled and tested. Such AAV designs, dubbed selector AAV vectors, seek enriching for cells coedited through at a primary target locus and *ATP1A1* alleles that confer resistance to ouabain upon HDR-mediated acquisition of specific polymorphisms. Importantly, besides enriching for gene KI cell populations, combining selector AAV vectors with ouabain selection triggered the elimination of HDR-independent edits and off-target and/or random AAV donor DNA insertions from said populations. This selector AAV principle was successfully applied for inserting whole transgenes at safe harbor genomic loci as well as for tagging endogenous proteins. Further, selector AAV vector titration experiments revealed that the highest fold-enrichment factors of gene-targeted cell fractions were associated with the lowest vector input amounts. This feature might become beneficial for alleviating both AAV production costs and detrimental P53-dependent DDR activation. Of note, the dual viral vector platform described in **Chapter 4** was put to use for

streamlining and expanding selector AAV vector testing into hard-to-transfect primary cells.

As covered in **Chapter 2** and **Chapter 3**, there is a growing realization that, especially in DNA damage sensitive stem cells, programmable nuclease-induced DSBs are detrimental to target loci stability [22,23], and cell viability [7,19]. Significantly, when compared with Cas9 nucleases, $Cas9^{D10A}$ nickases are notoriously less mutagenic at both on-target and off-target genomic sequences as determined by reporter and unbiased genome-wide high-throughput assays, respectively [24,25]. $Cas9^{D10A}$ nickases further display greatly reduced P53-dependent DDR activation when tested side-by-side with their Cas9 nuclease counterparts [9,26]. In this context, transduction experiments described in **Chapter 5** have shown that combining a particular selector AAV design, dubbed in-linkage selector AAV, with Cas9 or $Cas9^{D10A}$ exposure leads to a significant increase in the frequencies of gene-targeted cells. The ouabain-dependent enrichment factor for gene-targeted cells was *circa* 7.5-fold higher when using $Cas9^{D10A}$ nickase delivery. Importantly, combining in-linkage selector AAV transduction with Cas9 nuclease or $Cas9^{D10A}$ nickase delivery led to the thorough elimination of donor DNA inserted at off-target genomic positions from genome-edited cell populations. Therefore, these proof-of-principle experiments indicating that selector AAV-based genome editing is transportable to protocols involving $Cas9^{D10A}$-induced HDR are relevant for furthering the refinement and application of DSB-independent cell engineering strategies.

In conclusion, through the investigation and integration of distinct viral vectors and RNA-programmable Cas9 proteins, the work presented in this thesis provides new insights and toolboxes for advancing genome editing through the improvement of performance aspects related to its efficiency, specificity and fidelity.

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