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

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Chapter 1

General introduction

General introduction and outline of this thesis

Nowadays, advanced tools deployed for genome editing (GE) mainly derive from the CRISPR/Cas9 system found to be in 2012 as a prokaryotic RNA-guide antiviral system. Typically, Cas9 proteins or engineered Cas9 proteins, *e.g.*, nucleases, can make endogenously site-specific double-stranded DNA breaks (DSBs) which are predominantly repaired via the non-homologous end joining (NHEJ) DNA repair pathway leading to the introduction of mutagenic insertions/deletions (indels). Hence, NHEJ-mediated GE can lead to the removal of pre-existing genetic information, *i.e.*, knock-out (KO). Alternatively, in the presence of exogenous donor DNA templates, *e.g.*, a transgene cassette flanked by DNA sequences identical to those present around the targeted DSB made by engineered RNA-guided nucleases, can result in gene knock-in (KI) through the homology-directed repair (HDR) DNA repair pathway. Differently from DSBs made by RNA-guided Cas9 nucleases, there are Cas9 protein variants that induce instead single-strand DNA breaks (SSBs), or nicks, by virtue of having the D10A or H840A mutations that result in the catalytic inactivation of their RuvC or HNH nuclease domains, respectively. Significantly, in contrast to DSBs, nicks are not substrates for error-prone NHEJ processes. Furthermore, nicks caused by nicking Cas9 proteins, *i.e.*, nickases, can also serve as stimuli for HDR in the presence of exogenous donor DNA templates for the purpose of achieving KI at target chromosomal sites. Ideally, SSB-based KI strategies can alleviate or even avoid the buildup of indels caused by DSBs, and hence it can be regarded as a preferable GE strategy choice in the regards to therapeutical applications.

In **Chapter 2**, the pros and cons of GE involving DSB-based homology-directed repair are elaborated. We discuss the pressing need for the development and application of less mutagenic GE procedures, namely, via using DSB-independent nickases which, as aforementioned, lead only to residual amounts of mutagenic indels and can be tailored together with matched donor DNA templates for precise SSB-mediated HDR. As such, SSB-mediated HDR can serve as a valuable approach for editing

therapeutically relevant cells, *e.g.*, induced pluripotent stem cells (iPSCs) and T cells, leading to the manufacturing of potentially safer cell products for transplantation purposes. In **Chapter 3**, we further elaborate on the use of nickase variants in the form of base editors directed at therapeutic GE applications. In particular, we commented on the work conducted by Chai *et al.* (Mol. Ther. Nucleic Acids. 2023 32:522-535), investigating repair of defective *DMD* alleles causing Duchenne muscular dystrophy (DMD) via adeno-associated viral (AAV) vector delivery of *trans*-splicing base editors *in vitro* and *in vivo* settings. Instead of testing AAV/CRISPR-Cas9-based *DMD* reading frame restoration, associated with the potentially prevalent capture of Cas9-encoding AAV at on- and off-target nuclease sites, the authors investigated dual AAV delivery of *trans*-splicing adenine-base editor constructs yielding a Cas9 nickase fused to an adenine deaminase that, together with a cognate gRNA, mediates adenine (A) to guanine (G) substitutions. The resulting substitutions disrupt splice site motifs leading to exon skipping of *DMD* exon sequences bearing premature stop codons. On the top of overcoming the limited cargo capacity of AAV vectors (*i.e.*, 4.7 kb), such dual AAV method provides for a DSB-free *DMD* reading frame correction option for treating DMD patients whose disease is caused by out-of-frame deletions.

Even though, conceptually, one expects installing desired therapeutic gene edits with the aid of different GE tools, it has become patently clear the challenge of achieving efficient and safe delivery of the necessary tools into target cells, especially those cells resistant to transfection or relevant to therapeutical applications. Moreover, one needs to contend with the fact with the increasing precision of the most advanced GE tools there is a concomitant trend towards larger size increase in the associated machineries, which further hampers their delivery efficiency. Taken these delivery and safety aspects together, we reasoned that combining distinct engineered viral vectors lacking all viral genes could serve as sources of GE tools upon robust transduction of target cells. In particular, based on the complementary characteristics of

adenoviral (AdV) and adeno-associated viral (AAV) vectors we postulated that a robust and precise GE system could be assembled by combining the former and latter platforms for delivering CRISPR/Cas9 reagents and donor DNA templates into human cells, respectively.

Indeed, contrary to linear uncapped AAV genomes, terminally capped double-stranded AdV genomes are not prone to off-target DNA insertions making them suitable for the delivery of Cas9 enzyme constructs since it is especially important to prevent chromosomal integration of these constructs to minimize the buildup of off-target effects. In addition, also in contrast to AAV vectors, AdV vectors can accommodate full-length Cas9 constructs together with single or multiple gRNAs. Conversely, AAV genomes are proficient substrates for HDR possibly owing to the AAV DNA structure featuring secondary-structured inverted terminal repeats flanking single-stranded DNA. Besides allocating AdV and AAV systems for the delivery of, respectively, Cas9 constructs and distinct types of donor DNA templates, the role of distinct AAV donor DNA structures on the efficiency and accuracy of genome editing was investigated. Hence, in **Chapter 4**, we put forward and systematically evaluate different iterations of this dual viral vector system in HeLa cells, human mesenchymal stem cells (hMSCs) and skeletal muscle progenitor cells (myoblasts) in terms of their efficiencies, specificities, and accuracies. In this context, we extend earlier observations showing that 3rd generation, fully viral gene-deleted AdV vectors have a notoriously dampened cytotoxicity profile when compared to that of their 2nd generation counterparts.

In **Chapter 5**, we build on the dual viral vector platform introduced in **Chapter 4** by investigating its compatibility with a marker-free co-selection system for selecting gene-edited cells and, simultaneously, purging imprecisely edited cells via ouabain selection. The main sub-unit of the ubiquitously expressed sodium/potassium pump (Na^+/K^+ ATPase) is encoded by *ATP1A1* whose ATPase product is responsible for many physiological

functions including osmotic regulation. Interestingly, specific point mutations in *ATP1A1* conferring ouabain resistance are naturally found in the human population without disrupting the regular physiological functions of the Na^+/K^+ ATPase. Hence, aiming at improving the performance of AAV-based gene editing procedures, we sought to investigate AAV donor constructs harboring marker-free co-selection components (selector AAV vectors) permitting ouabain-dependent enrichment for genome-edited cells. We demonstrate that combining selector AAV vectors with ouabain treatments, in addition to enriching for genome-edited cell populations, eliminates imprecise on-target edits and off-target and/or random donor DNA insertions from said populations. Importantly, selector AAV vector titration experiments showed that the highest fold-enrichment factors for genome-edited cell fractions are associated with the lowest vector input amounts. This finding is expected to be beneficial for alleviating AAV vector production costs, off-target donor insertions and P53-dependent activation of the DNA damage response linked to AAV DNA, which is known to be particularly deleterious in stem cells with scientific and therapeutic relevance, *e.g.*, induced pluripotent stem cells and hematopoietic stem cells.