Adenoviral vectors as genome editing tools : repairing defective DMD alleles

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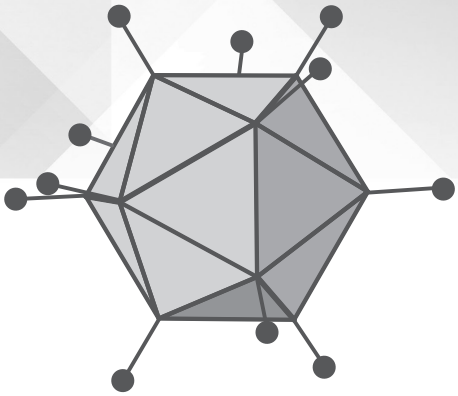

Conclusions and final remarks

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Adenoviral vectors (AdVs) constitute powerful gene delivery vehicles. However, so far, their potential for genome editing has not been extensively investigated. By tailoring AdVs as carriers of designer nuclease and donor DNA sequences, the research presented in this thesis expands the utility of the AdV platform in genome editing and provides insights for designing and testing new therapeutic options for tackling genetic disorders such as Duchenne muscular dystrophy (DMD).

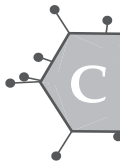
The data presented in **Chapter 2** demonstrate that fiber-modified AdVs are valuable delivery vehicles for introducing the RNA-guided nucleases (RGNs) components into target cell nuclei. These components, derived from the *S. pyogenes'* CRISPR/Cas9 system, consisted of single guide RNA (gRNA) and Cas9 molecules. Co-transduction of target cells with AdVs encoding gRNAs and Cas9 nuclease resulted in high-frequency site-specific DSB formation and recruitment of the non-homologous end joining (NHEJ) pathway yielding targeted mutagenesis in different human somatic cell types, including non-transformed cells. Therefore, **Chapter 2** sets the stage for expanding the range of experimental systems in which RGNs can be tested and optimized.

In addition to introducing designer nuclease coding sequences, in **Chapter 3**, AdVs are also shown to be useful for delivering donor DNA sequences for homologous recombination (HR)-mediated gene targeting. Importantly, the work presented in **Chapter 3** demonstrates that the nature of donor DNA templates greatly affects the specificity and accuracy of designer nuclease-assisted genome editing. In particular, when compared to other donor DNA structures (i.e. free-ended and covalently closed), protein-capped templates delivered in the context of AdV genomes were shown to markedly reduce off-target and inaccurate insertions of the exogenous DNA. Therefore, these findings are particularly relevant for HR-mediated genome editing strategies aiming at high-fidelity genetic modifications of human cells.

In **Chapters 4** and **5**, it is established that AdVs encoding designer nucleases can be tailored for achieving efficient and robust template-free *DMD* repair in muscle progenitor cells derived from patients with DMD. These approaches exploit the fact that, when compared to HR, NHEJ is preferentially used as DNA repair pathway in mammalian cells. Of note, the correction of faulty endogenous *DMD* alleles, ultimately, leads to the synthesis of shorter, but functional, dystrophins reminiscent of those underlying milder Becker muscular dystrophy phenotypes. Furthermore, the *de novo* dystrophin synthesis is permanent and under the control of the native regulatory elements in *DMD*-edited cells. The research presented in **Chapter 4** demonstrates, in particular, that AdV-mediated transduction of designer nucleases permits correcting defective *DMD* alleles through reading frame resetting, DNA-borne exon-skipping and targeted deletions of reading frame-disrupting exons.

These findings are expanded in **Chapter 5** by reporting that AdVs encoding RGN multiplexes can restore the *DMD* reading frame by triggering short- and long-range excisions of DNA segments encompassing out-of-frame exons. Notably, by removing the over 500-kb major *DMD* mutational hotspot, AdVs encoding RGN multiplexes can be tailored for tackling more than 60% of the reported DMD-causing mutations via a single gene-editing strategy (**Chapter 5**). Taken together, the data presented in **Chapters 4** and **5** establish AdVs as a versatile gene-editing platform for robust *DMD* reading frame restoration. Importantly, AdV-based *DMD* editing led to the rescue of dystrophin synthesis in target cell populations without the need for implementing expedients to select for *DMD*-edited cells or nuclease-exposed cell fractions.

Taken together, these findings are expected to aid the designing and testing of new genetic therapeutic interventions for tackling DMD. For instance, towards a clinical translation, the implementation of fiber-modified AdVs for targeting human cells with myoregenerative capacity will allow for assessing wanted and unwanted gene editing outcomes in the context of *DMD* mutations that are not currently represented by the available animal models. Such research will benefit from the development and application of sensitive and unbiased assays for detecting DSBs at a genome-wide level as well as from ongoing research aiming at isolating, characterizing and testing human primary cells with myoregenerative capacity. The latter entities are particularly enticing in that they represent the natural cellular targets for testing *ex vivo* gene editing concepts aiming at addressing the pathological aspects underlying different muscular dystrophies. Moreover, still related to future research efforts, it will also be important to improve HR-mediated genome editing strategies for restoring the full-length *DMD* coding sequence. In addition to *DMD* editing, the integration of AdV and genome editing technologies is expected to be extrapolated to other genetic disorders caused by loss-of-function mutations generated by premature stop codons or aberrant splicing, such as those underlying certain forms of β-thalassemia.



In conclusion, designer nuclease-assisted genome editing is opening an enlarged set of basic and applied research opportunities, from shedding light on human biology to developing new gene therapies. However, the clinical application of genome editing technologies will be crucially dependent on further advances so that safety concerns are thoroughly addressed, for instance by assessing the immunogenicity of nuclease components and the long-term effects caused by genome modification events. Furthermore, the prospect of introducing permanent changes in the germline, gives rise to ethical issues that need to be thoroughly discussed by the scientific community and by society at large. Therefore, it is to be expected that future genetic therapies for monogenetic disorders in general, and for DMD in particular, will focus on gene editing of somatic cells.