Shepherding precision gene editing with CRISPR-Cas9 variants and adenoviral vectors

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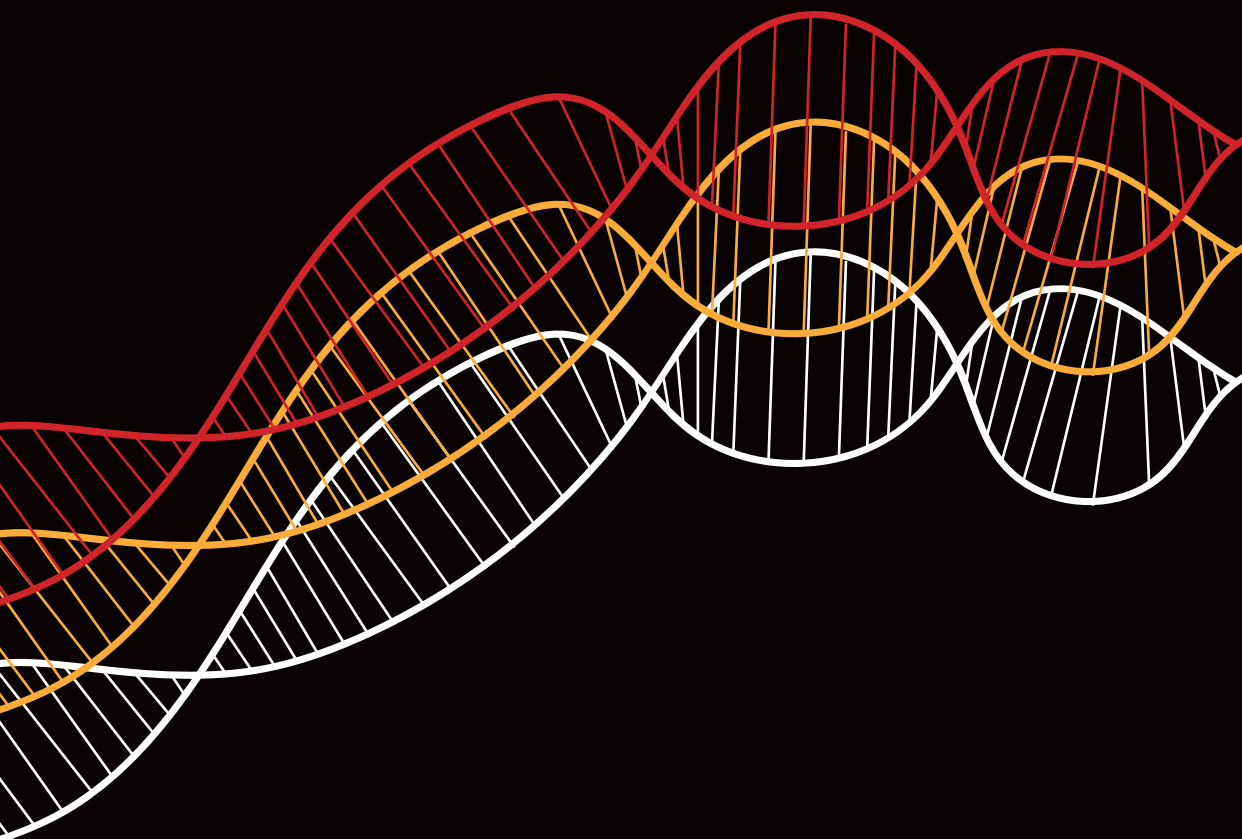
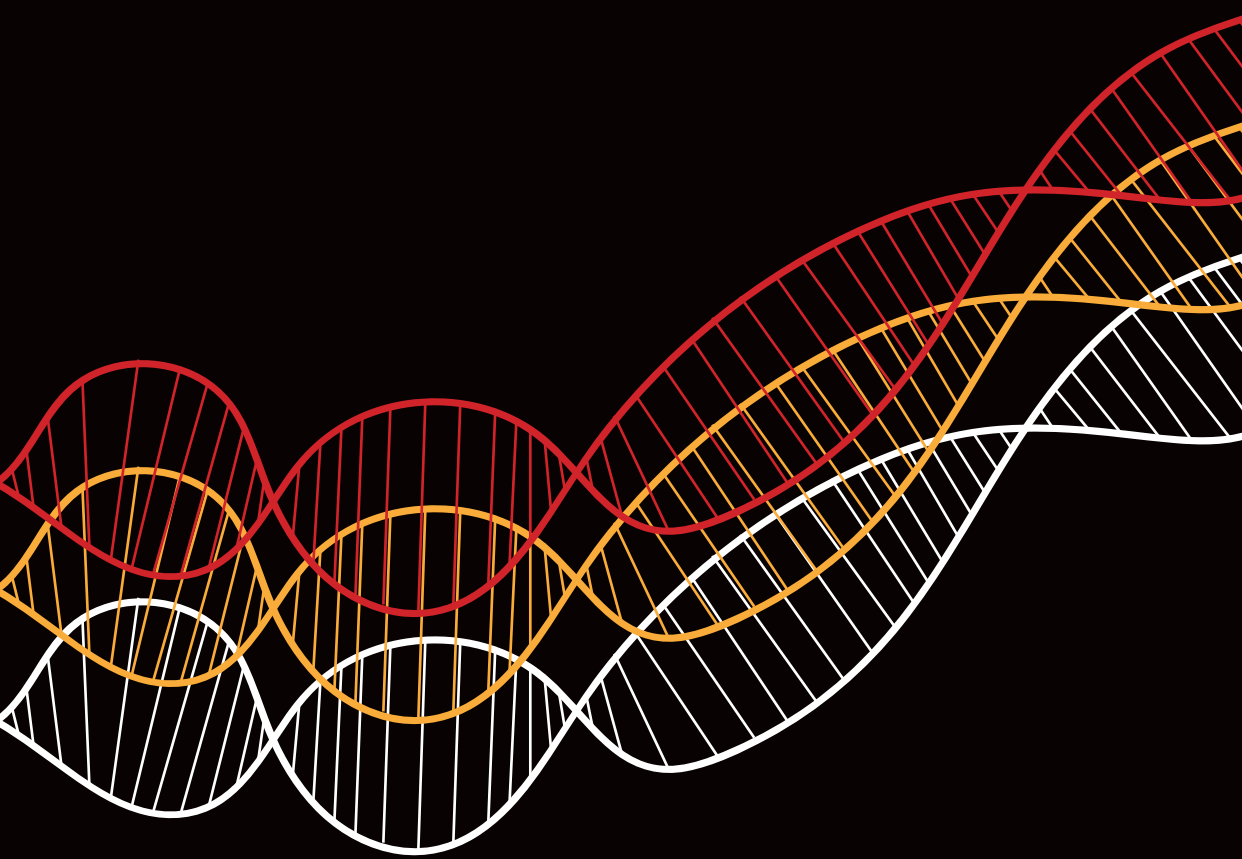


Chapter 6

Conclusions and Final remarks



Conclusions and Final remarks

Genome editing (GE) strategies based on homology-dependent and homology-independent DNA repair pathways activated by programmable nucleases, e.g., RNA-guided nucleases (RGNs) from CRISPR-Cas systems, permit modifying specific chromosomal sequences in living cells, spanning from single base-pairs to whole genomic tracts. This set of techniques is already proving its outstanding potential in a variety of fields, ranging from basic research to applied biotechnology, biomedical research, and medicine. Nevertheless, constant efforts are in demand to further improve the efficiency and specificity of GE tools and procedures, especially those directed at clinical translation. As outlined in **Chapter 2**, key to broaden the possibilities of GE technologies to gene and cell therapies, is their application in stem cells. To this end, it is essential to implement delivery systems that permit introducing, in an efficient and non-cytotoxic manner, the latest-generation GE tools into hard-to-transfect target cell types (e.g., non-transformed somatic cells and human induced pluripotent stem cells, hiPSCs). It is in this context that, in **Chapter 3** and **Chapter 4**, high-capacity adenoviral vector particles (AdVPs) are investigated as carriers of GE tools consisting of conventional and novel programmable nucleases alone or together with donor DNA sequences prone to specific DNA repair pathways. The versatility of this delivery platform facilitated the testing of novel GE approaches in diverse stem and progenitor cells with therapeutic potential, including hiPSCs and muscle progenitor cells. Specifically, the strategies were tailored to correct the genetic defect underlying Duchenne Muscular Dystrophy (DMD). DMD is a fatal X-linked muscle wasting disorder caused by a broad range of loss-of-function mutations in the largest known protein-coding gene in the human genome, i.e., the ~2.4 Mb-long dystrophin-encoding *DMD* gene. To date, DMD-targeted gene therapies focus mainly on the overexpression of microdystrophins upon adeno-associated viral vector delivery or *in situ* assembly of Becker-like dystrophins after RGN transfer followed by non-homologous end joining (NHEJ)-mediated restoration of defective *DMD* reading frames.

In fact, **Chapter 3** explores the use of AdVPs for multiplexing GE approaches aiming at NHEJ-mediated repair of the *DMD* reading frame and ensuing expression of Becker-like dystrophins upon targeted DNA deletion. The multiplexing GE strategy investigated in this Chapter is based on the coordinated formation of DSBs by covalently joined RGN pairs (i.e., forced RGN heterodimers) designed for the excision of *DMD* reading frame-disrupting mutations. Uncoordinated activity of independently acting RGNs proved, in this study and elsewhere, to perform at diverse levels of efficiency and to generate substantial amounts unintended genomic modifications that compound the intended GE outcome in the form of precise DNA deletions. In **Chapter 3**, by employing AdVPs to deliver forced RGN heterodimers, it is uncovered that the frequency and accuracy of targeted DNA deletions are superior to that obtained when the various RGN components are delivered separately. This approach bodes well for GE applications in which generating targeted chromosomal deletions in a precise and efficient manner is necessary.

Chapter 4 expands on the research efforts described in **Chapter 3** by further leveraging the AdVP platform to, in this case, recruit homology-dependent DNA repair processes to achieve long-term complementation of DMD-causing mutations regardless of their type or location, namely, via site-specific chromosomal insertion of transgenes expressing the full-length striated muscle-specific isoform of dystrophin (427 kDa). The AdVP-based GE strategies investigated in **Chapter 4** involved the delivery of RGNs together with donor DNA templates prone to homologous recombination (HR) or homology-mediated end joining (HMEJ). HMEJ and HR donor constructs were both designed for targeted chromosomal integration of full-length dystrophin expression units at the commonly used "safe harbour" locus *AAVS1* at 19q13.42. Generally, it was found that the delivery of HMEJ donors led to higher frequencies of on-target integrations than those resulting from the transfer of HR donors. The efficiencies of the HMEJ- and HR-based GE strategies were instead found to be similar in hiPSCs yet, interestingly, DSB-induced gene targeting levels under p53 inhibiting conditions could be rescued specifically in hiPSCs transduced with HMEJ donors. In conclusion, the AdVP methodologies described in **Chapter 4** allow the investigation and application of different gene knock-in approaches in hard-to-transfect human cell types irrespective of RGN complex and transgene sizes. Altogether, the work described in **Chapter 3** and **Chapter 4** support the use of AdVPs for the development of effective and broadly applicable gene therapies based on CRISPR components, including those involving *ex vivo* correction and autologous transplantation of stem/progenitor cells. Nevertheless, both studies also highlight drawbacks of DSB-based GE strategies in the form of undesired on-target and off-target genomic modifications and, therefore, stress the need to carefully scrutinise GE outcomes when applying such strategies. Additionally, the data presented in **Chapter 4** further support the argument that DSB-based GE is particularly impaired in stem cells likely due to the induction of p53-dependent cell cycle arrest and apoptosis by the programmable nucleases. In light of these by-product events consistently detected in cells exposed to programmable nucleases , recent developments on genomic engineering comprise the progression from chromosomal cutting to chromosomal non-cutting approaches based on nicking CRISPR-Cas variants.

Contributing to this line of research, in **Chapter 5**, the benefits of employing a DSB-free GE strategy to modify particularly sensitive genomic regions and cells is instead investigated. This *in trans* paired nicking strategy, based on the simultaneous formation of single-strand DNA breaks (SSBs) at donor and acceptor DNA by CRISPR–Cas9 nickases, proved successful in knocking-in large DNA segments efficiently and precisely at loci associated with haploinsufficiency and essentiality in diverse human cell types, including hiPSCs. Moreover, this SSB-based GE strategy circumvents most large- and small-scale mutagenic events caused by DSBs maximizing the preservation of cellular genotypes and phenotypes. Hence, the seamless and scarless character of *in trans* paired nicking might be particularly beneficial in the editing of stem cells, especially when precise and predictable genetic interventions are

delivery of forced RGN heterodimer components which, in comparison to split conventional RGN multiplexes, engage target sequences in a more coordinated fashion. Moreover, besides serving as probes for investigating different gene targeting approaches independently of GE tool and donor DNA sizes, AdVPs constitute a robust platform for delivering and stably installing in a targeted homology-dependent manner large genetic payloads in human cells. Finally, closely monitoring GE procedures and further progressing towards DSB-free GE strategies will become ever-more crucial in aiding gene therapy research progressing to clinical application. In this context, tailoring AdVPs for the testing of such precision GE approaches in therapeutically relevant cell types and animal models can contribute to opening the doors to the development of safer therapeutic interventions that tackle the root cause of genetic disorders, such as DMD.