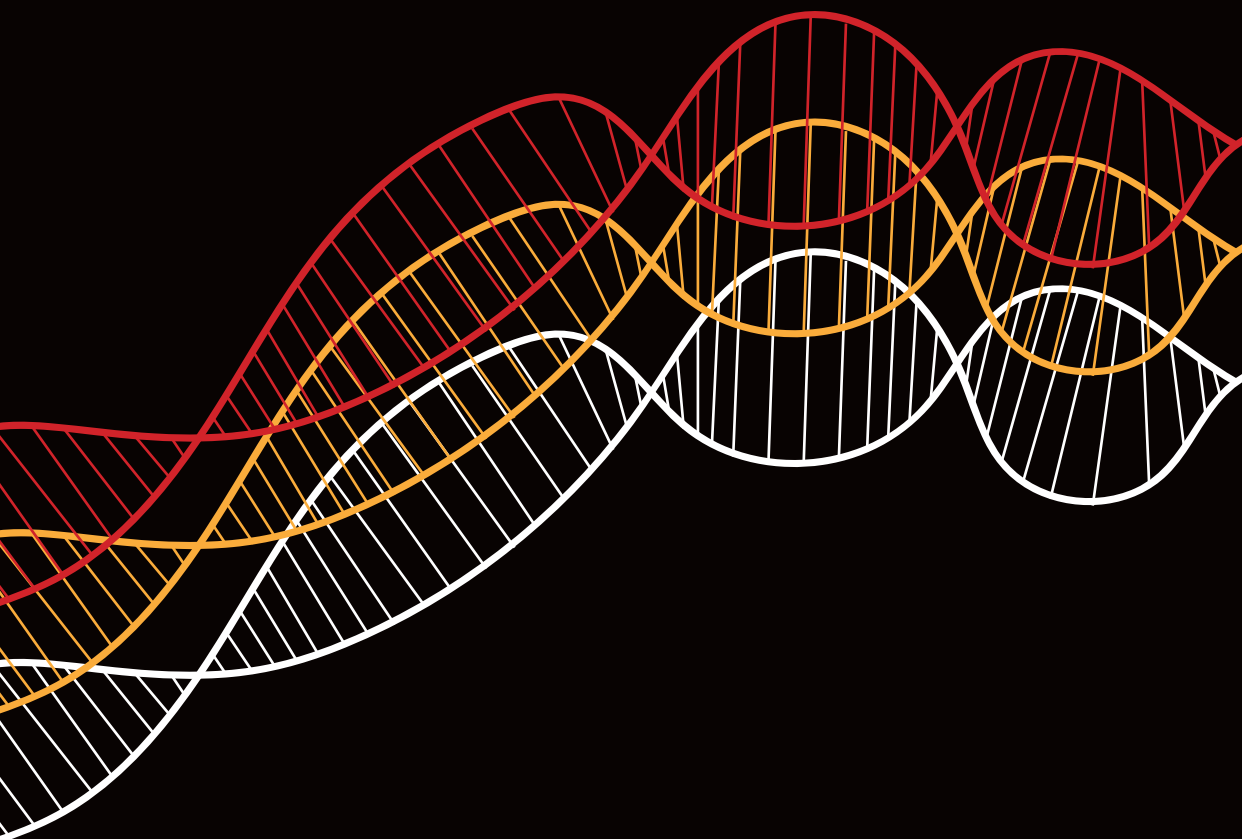
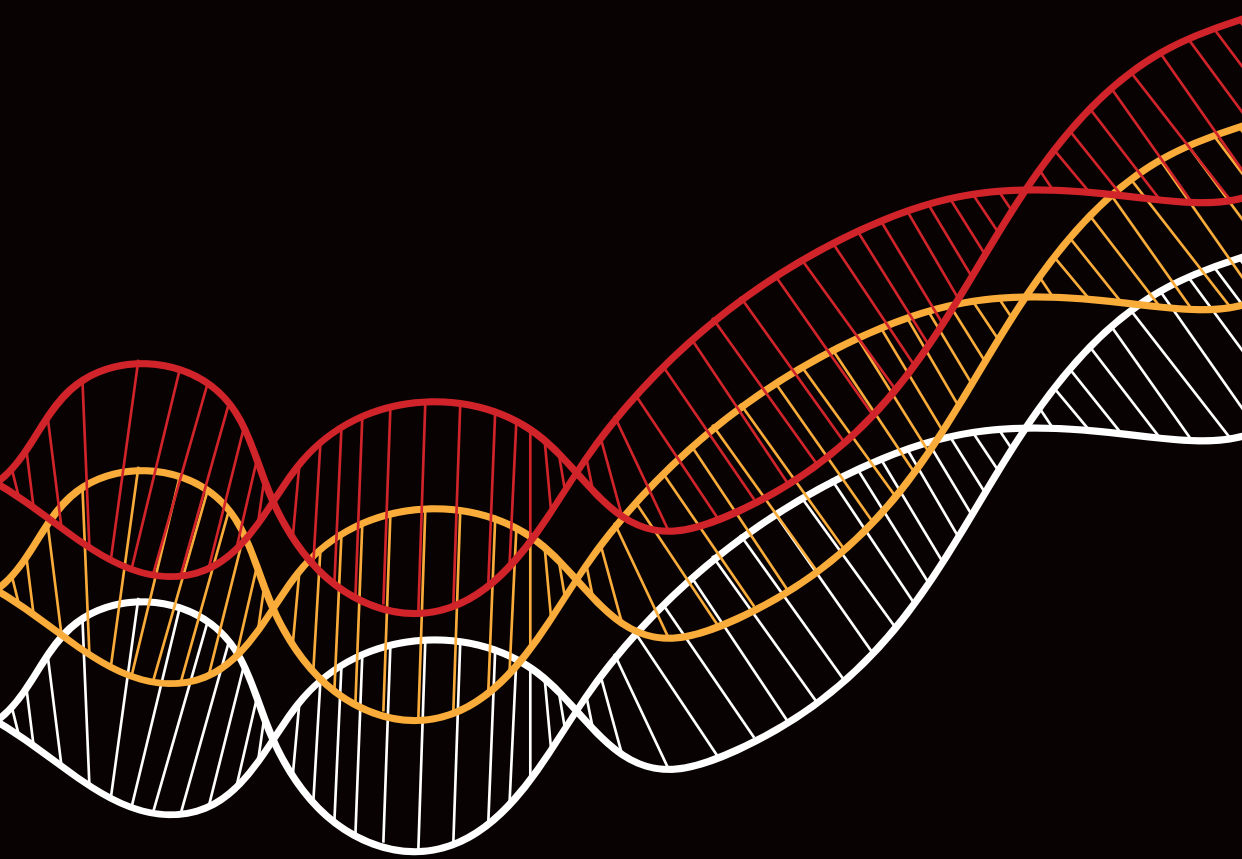
Shepherding precision gene editing with CRISPR-Cas9 variants and adenoviral vectors

Tasca, F.



Chapter 1

General Introduction



General Introduction

The past two decades have seen the growing development and consequent vast application of next-generation genome editing (GE) tools in fundamental and applied research. Nowadays GE based on RNA-guided nucleases (RGNs) (e.g., engineered CRISPR-Cas9 nucleases) are the most common tools for targeted genetic modification of eukaryotic cells. Normally, RGN-induced double-stranded DNA breaks (DSBs) are employed with or without donor DNA constructs to obtain specific GE goals through targeted DSB repair involving homology-directed repair (HDR) or non-homologous end joining (NHEJ) pathways, respectively. Nevertheless, GE technologies are in need of increased efficiency and accuracy, especially looking forward to translation into diverse clinical applications. The work presented in this thesis focuses on investigating new principles for improving the efficiency and accuracy of GE, particularly in cells with high therapeutic potential, such as induced pluripotent stem cells (iPSCs). In particular, these principles entailed (i) modifying the structure of donor HDR substrates and RGNs, and (ii) integrating third-generation adenoviral vector and RGN technologies. **Chapter 2** provides an updated review on the initial efforts and recent progress in the field of GE and adenoviral vector systems. Moreover, **Chapter 2** outlines the use of the latter gene delivery tools for GE of human stem cells and their progeny towards the fulfilment of their therapeutic potential.

Despite noticeable advances in the GE field, described in **Chapter 2**, several hurdles still hinder its full potential. The work described in **Chapter 3** and **Chapter 4** is based on employing fully viral gene-deleted adenoviral vectors (*aliases* third-generation or high-capacity adenoviral vectors) as delivery agents of modified donor HDR substrates and/or RGNs whose combined effects result in improved efficiency and specificity of NHEJ- and HDR-mediated GE strategies (**Chapter 3** and **Chapter 4**, respectively). In **Chapter 3**, it is established that Cas9 heterodimer fusion proteins achieve precise deletion of specific chromosomal DNA stretches in a more controlled manner than conventional, independently acting, Cas9 monomers. With the strategy developed in **Chapter 4** it was instead possible to obtain large-scale (up to 14.6 kb) precise gene knock-ins in hard-to-transfect stem and progenitor cells when using a third-generation adenoviral vector system for the combined delivery of donor DNA templates and high-specificity CRISPR-Cas9 nucleases. Together, **Chapter 3** and **Chapter 4**, highlight the versatility of converting third-generation adenoviral vectors into delivery agents of GE tools and applying these in diverse therapeutically relevant cell types, i.e., iPSCs and myoblasts. The insights derived from these studies were obtained in the context of research directed at treating Duchenne muscular dystrophy (DMD).

DMD is a progressive muscle-wasting disease caused by mutations in the vast (i.e., circa 2.4-Mb) dystrophin-encoding *DMD* gene. In most cases, DMD-directed gene therapies aim at the production of partially functional dystrophins, either through mutation-specific DMD repair strategies resulting in the expression of Becker-like dystrophins, or the overexpression of microdystrophins. In **Chapter 3**, the investigated NHEJ-mediated multiplexing GE

strategy, based on Cas9 heterodimer fusion proteins and coordinated action of the resulting RGN pairs, is applied to repair defective DMD alleles resulting in the expression of Becker-like dystrophins. In **Chapter 4**, stable expression of fully functional dystrophin molecules upon targeted insertion of full-length dystrophin expression units into a "safe harbor" chromosomal locus is instead investigated to achieve complementation of DMD-causing mutations regardless of their type or location.

Finally, **Chapter 5** stresses the need to further enhance the specificity and fidelity of GE procedures and explores the benefits of progressing towards DSB-free GE strategies. The work detailed in this chapter focuses on the use of a single-strand DNA break (SSB)-based GE strategy (i.e., *in trans* paired nicking) to edit particularly sensitive genomic regions and cells. This SSB-based GE strategy proved to be successful in making seamless edits and potentially expands the editable genome to tracts previously not possible to modify effectively due to their repetitive nature and/or essentiality for proper cell function or viability.

All together the work presented in this thesis broadens the horizon of possible GE applications, including those directed at gene therapies, by investigating the feasibility of using adenoviral vectors to test novel GE approaches and by exploring the utility of an emerging DSB-free GE strategy with a seamless and scarless character.