Adenoviral vectors as genome editing tools : repairing defective DMD alleles

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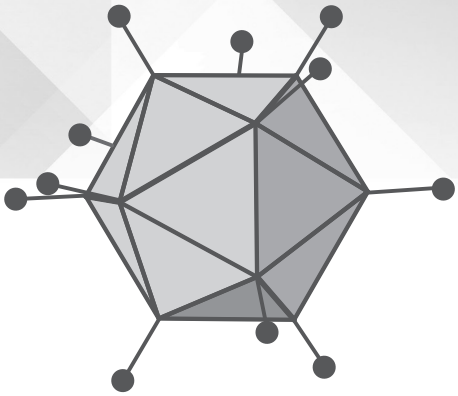

Addendum

Contents *Addendum*

Summary

Genome editing entails the manipulation of specific DNA sequences in the genome of living cells. In the last couple of decades, remarkable advances in genome editing have been made possible by the development of sequence-specific programmable nucleases such as zinc-finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs) and, more recently, RNA-guided nucleases (RGNs). The value of programmable nucleases arises from their ability to induce site-specific double-stranded DNA breaks (DSBs) triggering endogenous DNA repairs pathways, which are ultimately exploited for achieving permanent and targeted genetic modifications. In mammalian cells, the two major DNA repair pathways are the non-homologous end-joining (NHEJ), a direct end-to-end ligation of DNA termini often resulting in the incorporation of small insertions and deletions (indels), and the homologous recombination (HR), a DNA synthesis-dependent DSB repair mechanism suitable for introducing precise genetic changes directed by a donor DNA template. **Chapter 1** of this thesis provides a review of the recent progress in the genome editing field with particular emphasis on the specificity and fidelity attainable by deploying DNA-editing procedures based on the delivery of programmable nucleases into mammalian cells. As discussed in **Chapter 1**, the delivery of the sizable and multicomponent gene editing tools into target cell nuclei represents one of the major bottlenecks that need to be overcome for advancing genome editing methodologies.

The "delivery issue" related to the introduction of CRISPR/Cas9-derived RGNs into human cells is addressed in **Chapter 2**. In particular, the work described in this chapter demonstrates that fiber-modified adenoviral vectors (AdVs) are valuable tools for introducing the coding sequences of the binary RGN components (i.e. Cas9 and gRNA) into a broad array of human cell types, including non-transformed cells. By deploying RGN complexes customized to target a recombinant model *eGFP* allele or the endogenous *AAVS1* "safe-harbor" locus, the frequencies of targeted DSBs achieved in AdV-transduced cell populations, ranged from 18% to 65% (**Chapter 2**). Hence, this work contributes to expanding the panel of experimental systems in which RGNs can be tested and optimized.

In **Chapter 3** another important question was investigated: the contribution of the donor DNA structure to the specificity and accuracy of the gene editing process. By applying TALENs and RGNs together with different donor DNA substrates (i.e. protein-capped, free-ended and covalently closed DNA), **Chapter 3** provides evidence for a crucial role of the structure of the donor DNA on the specificity and accuracy of HR-mediated gene targeting. In particular, protein-capped DNA

delivered by AdVs was shown to markedly reduce random and inaccurate insertion of exogenous DNA. Although the overall efficiency of HR-mediated gene targeting resulting from AdV delivery of protein-capped donor DNA was not superior to that achieved by using donor DNA presenting different structures (e.g. linear free-ended DNA delivered by integrase-defective lentiviral vectors), the vast majority of AdV-edited cells harboured nonetheless the intended on-target single-copy integrants leading to homogenous transgene expression in the genetically-modified target cell population. These findings are particularly relevant for genome editing approaches aiming at high-fidelity manipulation of mammalian genomes.

In **Chapters 4** and **5**, AdVs encoding nucleases were probed as genome editing tools for testing different template-free *DMD* repair strategies based on the activation and recruitment of the NHEJ. Loss-of-function mutations in the ~2.4 Mb dystrophin-encoding *DMD* gene causes a fatal X-linked muscle wasting disorder called Duchenne muscular dystrophy (DMD). To date, curative therapies for DMD are not available and, amongst other research lines, genome editing has been put forward as a potential therapeutic modality. However, delivery of designer nuclease components into target cells with myoregenerative capacity warrants further investigations. Towards addressing this issue, different AdV-based gene editing strategies were devised and tested for repairing faulty endogenous *DMD* alleles in myoblasts derived from patients with DMD. The NHEJ-mediated strategies tested in **Chapter 4** included: (i) incorporation of small insertions or deletions at out-of-frame sequences for reading frame resetting; (ii) knock-out of splice acceptors for DNA-borne exon skipping; and (iii) coordinate DSB formations with pairs of designer nucleases (multiplexing) for excising the intervening sequences containing reading-frame disrupting exons. Following AdV delivery of designer nucleases, these strategies allowed for high-level *DMD* correction (up to 37% repaired *DMD* templates) readily leading to the detection of dystrophin synthesis in bulk target cell populations without the need to select for *DMD*-edited cells or nuclease-exposed cell fractions (**Chapter 4**).

The findings presented in **Chapter 4** were complemented in **Chapter 5** by experiments using AdVs for delivering RGN multiplexes, in single vector particles, customized to remove one or more exons causing the disruption of the *DMD* reading frame. Both multiplexing strategies resulted in robust *DMD* reading frame restoration and readily led to dystrophin detection in the cell populations without implementing any selection schemes. Importantly, the data presented in **Chapter 5** provides evidence that AdVs encoding RGN multiplexes can restore *DMD* reading frames by removing the over 500-kb major *DMD* mutational hotspot in which more than 60% of the reported DMD-causing mutations are located. The delivery

of RGN multiplexes in single AdV particles presumably increase the efficiency of gene editing strategies by allowing synchronous and stoichiometric expression of the various nuclease components in target cell populations.

In **Chapter 6**, the role of AdVs as gene editing tools for NHEJ-mediated repair of defective *DMD* alleles is further discussed in the context of other viral vector-based *DMD* editing strategies.

In conclusion, tailoring AdVs for genome editing purposes is expected to aid in the designing and testing of new therapeutic interventions for tackling DMD and is anticipated to continue to expand to other genetic disorders.

Nederlandse samenvatting

Genoom-editing omvat het manipuleren van specifieke DNA sequenties in het genoom van levende cellen. In de afgelopen decennia is enorme vooruitgang in genoom-editing mogelijk gemaakt door de ontwikkeling van sequentie-specifieke programmeerbare nucleasen, zoals zinc-finger nucleasen (ZFNs), transcription-activator-like effector nucleasen (TALENs) en, recenter, RNA-guided nucleasen (RGNs). De waarde van deze programmeerbare nucleasen komt voort uit hun vermogen om locatie-specifieke dubbelstrengs DNA breuken (DSB's) te induceren. Dit leidt tot de activatie van endogene DNA reparatiemechanismen, welke uiteindelijk worden benut om permanente en specifieke genetische modificaties aan te brengen in het genoom.

In zoogdiercellen zijn de twee voornaamste reparatiemechanismen van dubbelstrengs DNA breuken: non-homologous end-joining (NHEJ) en homologous recombination (HR). Bij NHEJ worden de twee DNA uiteinden rechtstreeks aan elkaar worden geplakt, dit gaat vaak gepaard met de incorporatie van kleine inserties en deleties (indels), terwijl het DNA-synthese-afhankelijke HR reparatiemechanisme geschikt is voor het aanbrengen van precieze genetische veranderingen met behulp van een donor DNA-template.

Hoofdstuk 1 van dit proefschrift geeft een overzicht van de recente ontwikkelingen op het gebied van genoom-editing, met de nadruk op de haalbare specificiteit en betrouwbaarheid van DNA-editing procedures gebaseerd op de aanlevering van programmeerbare nucleasen in zoogdiercellen. Zoals besproken in **Hoofdstuk 1**, is de aanlevering van het vaak grote en uit meerdere componenten bestaande gen-editing "gereedschap" in de doelcelkernen één van de grootste knelpunten die moeten worden overwonnen in genoom-editing technieken.

De "aanleveringskwestie" voor de introductie van CRISPR/Cas9 afgeleide RGNs in menselijke cellen wordt behandeld in **Hoofdstuk 2**. Het in dit hoofdstuk beschreven werk toont met name aan dat fiber gemodificeerde adenovirale vectoren (AdVs) waardevolle hulpmiddelen zijn voor het inbrengen van de binaire RGN componenten (d.w.z. Cas9 en gRNA) in een breed scala aan humane celtypen, waaronder niet-getransformeerde cellen. Door de inzet van RGN complexen ontworpen om een recombinant model *eGFP* allel of de endogene *AAVS1* "safe harbour" locus te bereiken, werden in AdV-getransduceerde cel populaties frequenties van gerichte DSB's bereikt variërend van 18% tot 65% (**Hoofdstuk 2**). Dit werk draagt hiermee bij aan de uitbreiding van experimentele systemen waarin RGNs kunnen worden getest en geoptimaliseerd.

In **Hoofdstuk 3** werd een andere belangrijke vraag onderzocht, namelijk

de vraag welke bijdrage de donor-DNA structuur levert aan de specificiteit en nauwkeurigheid van het gen-editing proces. Door TALENS en RGNs te gebruiken in combinatie met verschillende donor-DNA substraten (d.w.z. DNA waarbij de uiteinden zijn bedekt door eiwit (eiwitkap), onbedekte DNA uiteinden of covalent gesloten DNA), levert **Hoofdstuk 3** het bewijs voor een cruciale rol van de structuur van het donor-DNA in de specificiteit en nauwkeurigheid van HR gemedieerde gen aanpassing. Vooral via AdVs aangeleverd DNA met eiwitkap bleek willekeurige en onnauwkeurige insertie van exogeen DNA aanzienlijk te verminderen. De totale efficiëntie van HR-gemedieerde gen aanpassing d.m.v. AdV aangeleverd donor-DNA met eiwitkap was niet beter dan werd bereikt door donor-DNA met een andere structuur, bv. lineair DNA met vrije uiteinden aangeleverd door integrase-defecte lentivirale vectoren. Echter, de overgrote meerderheid van de door AdVs bewerkte cellen bevatte de beoogde single copy integratie op de juiste locatie, leidend tot homogene transgenexpressie in de genetisch gemodificeerde doelcellen. Deze bevindingen zijn met name relevant voor genoom-editing benaderingen gericht op een zeer nauwgezette manipulatie van zoogdier-genomen.

In **Hoofdstuk 4** en **5** werden AdVs coderend voor nucleasen gebruikt voor het testen van verschillende templateloze *DMD* reparatiestrategieën op basis van de activering van NHEJ. Verlies-van-functie mutaties in het ~2.4 Mb dystrofine-coderend *DMD*-gen veroorzaken een fatale X-chromosoom gebonden spieratrofie genaamd Duchenne spierdystrofie (DMD). Tot op heden zijn er geen therapieën voor DMD beschikbaar die leiden tot genezing; genoom-editing wordt als één van de benaderingen genoemd voor een potentiële therapeutische behandeling van deze ziekte. Echter, de aanlevering van de designer nuclease-componenten in doelcellen met myoregenerative capaciteit vergt verder onderzoek. Om dit probleem aan te pakken werden verschillende AdV-gebaseerde gen-editing strategieën bedacht en getest voor het repareren van defecte endogene *DMD* allelen in myoblasten afkomstig van patiënten met DMD.

De NHEJ-gemedieerde strategieën getest in **Hoofdstuk 4** omvatten: (i) de incorporatie van kleine inserties of deleties in out-of-frame sequenties om het leesraam te herstellen; (ii) knock-out van de splice acceptoren voor exon skipping op DNA niveau; en (iii) het creëren van gecoördineerde DSB formaties d.m.v. designer nuclease-paren (multiplexing) voor het verwijderen van de tussenliggende sequenties met leesraam verstorende exons. Deze strategieën leidden, na aanlevering van designer nucleasen via AdVs, tot een hoog niveau van *DMD* correctie (tot 37% herstelde *DMD* templates) en detectie van dystrofine synthese in de bulk van doelwitcellen zonder voorafgaande selectie van *DMD* gemodificeerde cellen of aan nuclease blootgestelde cel fracties (**Hoofdstuk 4**).

De bevindingen in **Hoofdstuk 4** werden in **Hoofdstuk 5** aangevuld met AdVs experimenten waarbij RGN multiplexen, ontworpen om één of meer exonen die de verstoring van het *DMD* leesraam veroorzaken te verwijderen, werden aangeleverd in één vectordeeltje. Beide multiplexing strategieën resulteerden in robuust *DMD* leesraam herstel en detectie van dystrofine in de cel populaties, zonder uitvoering van enige selectie.

Bovendien tonen de data in **Hoofdstuk 5** aan dat AdVs coderend voor RGN multiplexen het *DMD* leesramen kunnen herstellen door het voornaamste *DMD* mutatie gebied van meer dan 500 kb, waarin meer dan 60% van de gerapporteerde DMD veroorzakende mutaties liggen, te verwijderen. De aanlevering van RGN multiplexen via hetzelfde AdV deeltje verhoogt vermoedelijk de efficiëntie van gen-editing strategieën door synchrone en stoichiometrische expressie van de diverse nuclease-componenten in de doelcel populaties.

In **Hoofdstuk 6** wordt de rol van de AdVs als gen-editing gereedschap voor NHEJ-gemedieerde reparatie van defecte *DMD* allelen verder besproken in de context van andere virale vector-gebaseerde *DMD*-editing strategieën. Het op maat maken van AdVs voor genoom-editing doeleinden zal naar verwachting helpen bij het ontwerpen en testen van nieuwe therapeutische interventies voor de aanpak van DMD en zal zich hoogstwaarschijnlijk verder blijven uitbreiden naar andere genetische aandoeningen.

Curriculum Vitae

Ignazio Maggio was born on November 17, 1986 in Catania (Italy). During his bachelor studies (2005-2008) he performed a year of research internship under the supervision of Prof. Dr. Guido Scalia in the laboratory of Virology at the Policlinico of Catania, Italy. During this internship he worked on an experimental thesis about the detection of Polyomavirus JCV genomes in cerebrospinal fluids from patients with neurological diseases. In 2008, he obtained his BSc in "Biomedical laboratory technician" with first-class honours (110/110 *cum laude*) at the University of Catania, Italy.

The BSc study was followed by a 5-month internship in a national center for the diagnosis of chronic myeloid leukemia in Italy (Department of Biomedical Sciences, University of Catania). From 2009 to 2011, Ignazio attended the MSc program in "Molecular, Sanitary and Nutritional Biology, Curriculum Biomolecular Research" at the University of Urbino, Italy. During the last year of his MSc studies, he carried out his research internship under the supervision of Prof. Dr. Antonella Amicucci in the Department of Biomolecular Sciences at the University of Urbino. His MSc thesis described a genomic and transcriptomic approach for shedding light on the signal transduction pathways involved in the polarized hyphal growth in *Tuber*. In 2011 he obtained his MSc diploma with first-class honours (110/110 *cum laude*).

In May 2012, Ignazio started his PhD studies in the group of Prof. Dr. Rob C. Hoeben under the supervision of Dr. Manuel A.F.V. Gonçalves in the Department of Molecular Cell Biology at the Leiden University Medical Center (LUMC). The work performed during his PhD is presented in this thesis. Since May 2016 he has been appointed at the LUMC as a postdoctoral research fellow to work on a project on gene therapy for β-thalassemia.

PhD portfolio

Presentations at international conferences

20/10/2016 poster presentation — **Adenoviral vectors encoding CRISPR/Cas9 multiplexes rescue dystrophin synthesis in unselected muscle cell populations**, European Society of Gene and Cell Therapy and International Society for Stem Cell Research Collaborative Congress, Florence, Italy.

10-11/06/2016 poster presentation — **Adenoviral vectors encoding CRISPR-Cas9 nucleases for repairing *DMD* reading frame in unselected muscle cell populations,** Muscles2Meet, Neuromuscular Young Talent Symposium, Zeist, the Netherlands.

19/09/2015 oral presentation — **Adenoviral vector transduction of designer nucleases restores *DMD* reading frames in dystrophin-defective muscle cell populations**, European and Finnish Societies of Gene and Cell Therapy Collaborative Congress, Helsinki, Finland.

12/09/2015 poster presentation — **Genetic correction of DMD patient cells through genome surgery** (Genetische correctie van DMD patiënten cellen via genoom chirurgie), Prinses Beatrix Spierfonds Congress, Veldhoven, the Netherlands.

26/10/2014 oral presentation — **Adenoviral vector DNA is a preferred homologous recombination substrate for accurate genome editing using engineered nucleases,** XXII Annual European Society of Gene and Cell Therapy Congress in collaboration with the Netherlands Society of Gene and Cell Therapy, The Hague, the Netherlands.

06/09/2014 poster presentation — **Genetic correction of cells from patients with DMD** (Genetische correctie van cellen van patiënten met Duchenne), Prinses Beatrix Spierfonds Congress, Veldhoven, the Netherlands.

23/05/2014 oral presentation — **Adenoviral vector delivery of RNA-guided CRISPR/Cas9 nuclease complexes induces targeted mutagenesis in a diverse array of human cells**, 17th Annual Meeting of the American Society of Gene and Cell Therapy, Washington DC, USA.

13/03/2014 oral presentation — **Adenoviral vector delivery of RNA-guided CRISPR/Cas9 nuclease complexes induces targeted mutagenesis in a diverse array of human cells,** Netherlands Society of Gene and Cell therapy Spring Symposium, Lunteren, the Netherlands.

07/09/2013 poster presentation **Genetic correction of cells from patients with DMD** (Genetische correctie van cellen van patiënten met Duchenne), Prinses Beatrix Spierfonds Congress, Veldhoven, the Netherlands.

Attendance of scientific courses and conferences

29-31/08/2016 **Next Generation Sequencing data analysis**, MGC course, LUMC, Leiden, the Netherlands.

6/07/2016 **Indesign CS6**, Workshop for PhD-students and other researchers, Erasmus MC, Rotterdam, the Netherlands.

5/07/2016 **Photoshop and Illustrator CS6**, Workshop for PhD-students and other researchers, Erasmus MC, Rotterdam, the Netherlands.

11-15/01/2016 **Basic methods and reasoning in Biostatistics**, PhD course, LUMC, Leiden, the Netherlands.

26-30/10/2015 **Functional Imaging and Super Resolution**, PhD course, Erasmus MC, Rotterdam, the Netherlands.

29/09/2015 **GCP-WMO certificate** (Wet Medisch Wetenschappelijk Onderzoek met Mensen), LUMC, Leiden, the Netherlands.

17-20/09/2015 **European and Finnish Societies of Gene and Cell Therapy Collaborative Congress**, Helsinki, Finland.

27/05/2015 **Droplet digital PCR seminar**, Bio Rad, Utrecht, the Netherlands.

30/03-03/04/2015 **Modern DNA concepts and tools for safe gene transfer and modification**, EMBO Workshop, Evry, France.

26-27/01/2015 **Linux and basic scripting**, MGC course, Leiden, the Netherlands.

23-26/10/2014 **XXII Annual European Society of Gene and Cell Therapy Congress in collaboration with the Netherlands Society of Gene and Cell Therapy**, The Hague, the Netherlands.

21-24/05/2014 **17th Annual Meeting of American Society of Gene and Cell Therapy**, Washington DC, USA.

14-15/04/2014 **Epigenetic Regulation in Health and Disease**, MGC course, LUMC, Leiden, the Netherlands.

27-28/03/2014 **Genome Maintenance and Cancer**, MGC course, LUMC, Leiden, the Netherlands.

13/03/2014 **Netherlands Society of Gene and Cell therapy Spring Symposium**, Lunteren, the Netherlands.

10-14/06/2013 **Transgenesis, gene targeting and innovative mouse models**, MGC course, LUMC, Leiden, the Netherlands.

6-8/02/2013 **Technology Facilities (Genomics and Transcriptomics, Proteomics, Imaging)**, MGC course, LUMC, Leiden, the Netherlands.

Teaching training

25-28/11/2014 **Practical course on plasmid isolation and analysis**, teaching assistant, LUMC, Leiden, the Netherlands.

26-29/11/2013 **Practical course on plasmid isolation and analysis**, teaching assistant, LUMC, Leiden, the Netherlands.

27-30/11/2012 **Practical course on cloning of a DNA fragment from a virus**, teaching assistant, LUMC, Leiden, the Netherlands.

List of publications

Maggio I., Liu J., Janssen J.M., Chen X., and Gonçalves M.A.F.V. Adenoviral vectors encoding CRISPR/Cas9 multiplexes rescue dystrophin synthesis in unselected populations of DMD muscle cells. *Submitted manuscript*.

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