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Homology-directed safe harbor insertion of transgenes encoding full-length dystrophin through high-capacity adenoviral vector delivery of CRISPR-Cas9 nucleases and donor DNA

Introduction

Duchenne muscular dystrophy (DMD) is a severe X-linked muscle-wasting disease caused by a variety of mutations in the dystrophin encoding gene *DMD* that, spanning more than 2.2 Mb, is one of the largest human genes known. More then 1000 diverse mutation have been linked to DMD and several genetich theraphy approach are beeing investigated[1]. We aim to develop an all inclusive ex-vivo DMD genetic therapy involving knocking-in a "healthy" copy of the full-length dystrophin coding sequence (11 kb) into the *AAVS1* safe-harbour locus in human myogenic cells.

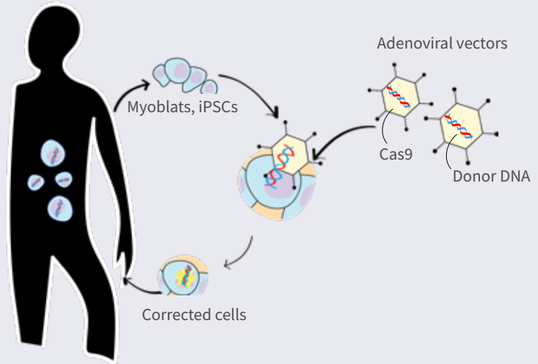

Figure 1. Schematich represantation of an adenoviral vectors-mediated ex-vivo genetich therahy.

Genome editing strategies

Standard and DSB[2] genome editing (GE) strategies targeting *AAVS1* safe harbor locus based on AdV.mCherry.Fl-Dys and AdV.mCherry.Fl-Dys.TS.

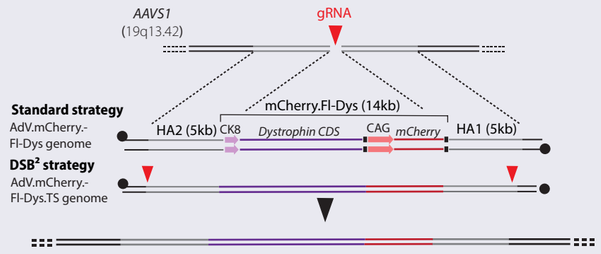

Figure 2. AdV.mCherry. Fl-Dys and AdV.mCherry.Fl-Dys.TS encode for mCherry reporter, under the control of the constitutively active CAG promoter, and full-length dystrophin (Fl-Dys), under the control of a muscle-specific promoter CK8, (mCherry.Fl-Dys; 14kb). Sequences corresponding to human *AAVS1* DNA flanking the CRISPR-Cas9 target site ("homology arms"; HA1 and HA2) are shown in gray (5 kb each). Red arrows, CRISPR-Cas9 target sites.

High-capacity adenoviral vectors

Main advantages[2,3]:
- Large packaging capacity (up to 36 Kb)
- High production yields (i.e. titers)
- Efficient transduction dividing and not dividing cells
- Episomal nature (i.e. non-integrating)
- High genetic stability
- Clinically applicable (e.g. vaccine and oncolytic agents)
- Amenable to tropism modifications

The **AdV.mCherry.Fl-Dys** tropism-modified vector efficiently transduce two indipentend patient-derived myoblats lines resulting in high levels of mCherry expression as evaluated through flowcytometry (a) and direct fluorescence microscopy (b). The tropism-modified **AdV.Cas9.gRNA^{AAVS1}** vector transduction resulted in relatively high levels of indels detection at the desired target site as depicted through T7EI-based genotyping assays (c-d).

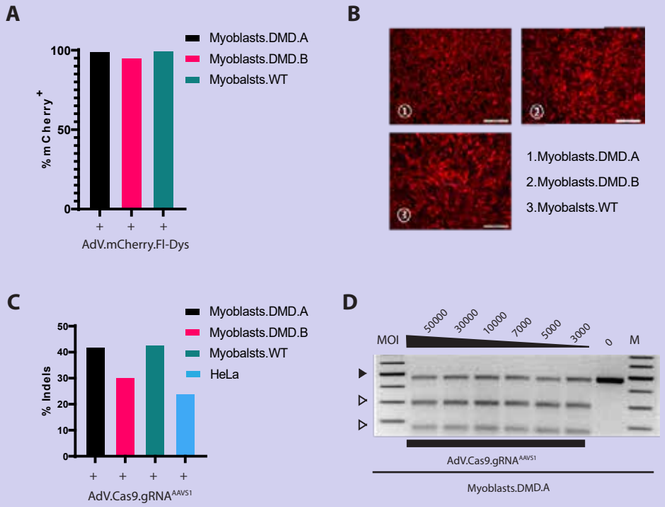

Figure 3. (**A**) Quantification of transduction levels through mCherry-directed flow cytometry at 3 days post transduction of patient-derived DMD.A, DMD.B and wild-type (WT) myoblasts with an AdV expressing the mCherry reporter at MOIs 20 HeLa-cell transducing units (HTU)/cell. (**B**) Direct fluorescence microscopy images depicting mCherry expression in DMD.A, DMD.B and WT myoblasts at 2 days post- transduction with AdV.mCherry.Fl-Dys at MOI 20 HTU/cell. (**C**) Targeted gene disruption frequencies determined by densitometry of the DNA fragments obtained through T7EI-based genotyping assay in DMD.A, DMD.B, WT myoblasts and HeLa cells treated with AdV.Cas9.gRNA^{AAVS1}at MOI 10000 genome copies (GC)/cell. (**D**) Representative agarose gel electrophoresis of *AAVS1*-specific PCR products amplified from chromosomal DNA of DMD.A myoblasts treated with AdV.Cas9.gRNA^{AAVS1} at indicated MOIs (GC/cell). Amplicons were treated with the mismatch-sensitive enzyme T7EI, upon denaturation and re-annealing.

Full-length dystrophin rescue

AdVs expressing mCherry reporter and full-length dystrophin under the control of a muscle-specific promoter (CK8) have been employed to carry out HDR-mediated GE experiments in two independent DMD patient-derived myoblast lines (i.e. DMD.A and DMD.B). Flow cytometry showed that almost 100% of the target cells could be transduced and express mCherry (Figure 4a-b). Subsequent sub-culturing and flow cytometry monitoring of stable transduction levels revealed that the **standard** GE approach, based on regular donor templates, led to **16% and 1% of integration levels** in DMD.A and DMD.B myoblasts, respectively. Importantly, the in trans paired breaking GE strategy (**DSB[2]**), based on CRISPR-Cas9 target site-flanked donor templates, led instead to a significant increase of integration levels, i.e., **64% and 6%** in DMD.A and DMD.B myoblasts, respectively (Figure 2a-b). Activation of the CK8 promoter and subsequent dystrophin expression has been detected through immunofluorescence microscopy upon differentiation of mCherry-positive DMD.A myoblasts into myotubes (Figure 2d).

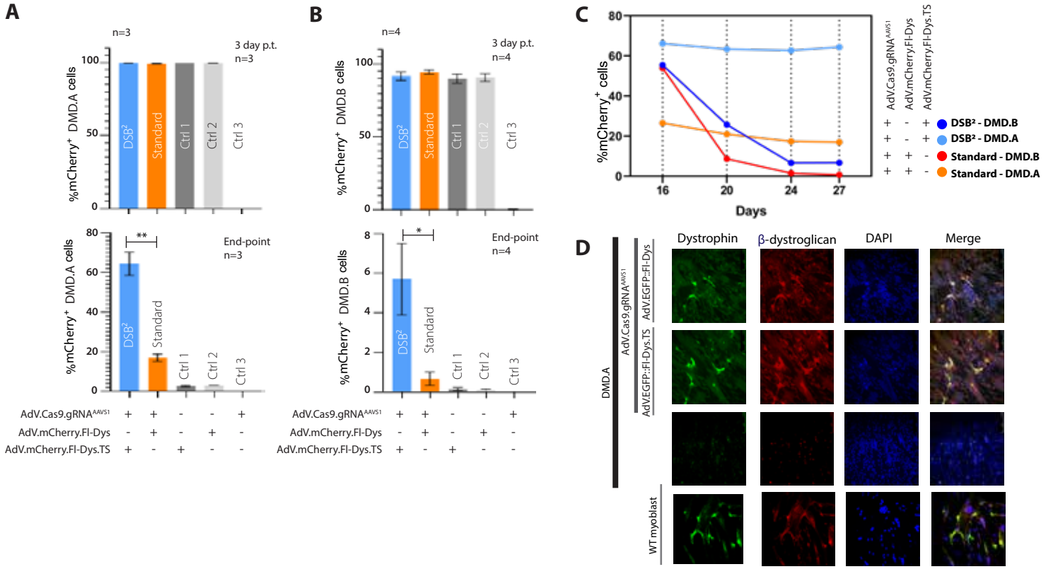

Figure 4. (**A-B**) Quantification of genetically modified DMD.A and DMD.B myoblasts. Flow cytometry of cell cultures upon co-transduction with AdV.mCherry.Fl-Dys or AdV.mCherry.Fl-Dys.TS and AdV.Cas9.gRNA^{AAVS1}. AdV.mCherry.Fl-Dys, AdV.mCherry.Fl-Dys.TS and AdV.Cas9.gRNA^{AAVS1} vector doses applied on DMD.A myoblats were, respectively, 500 HTU/cell, 500 HTU/cell and 10000 GC/cell; and on DMD.B myoblasts the vector doses applied were, 6 HTU/cell, 6 HTU/cell and 10000 GC/cell. Data were acquired at 3 days post-transduction (p.t.) (upper panel) and 27 days post-transduction (lower panel) and are presented as mean ± S.E.M of three (a) and four (b) biological replicates. Significance between the indicated datasets was calculated with t-test; **P< 0.002, *P< 0.033. (**C**) Monitoring genetically modified DMD.A and DMD.B myoblasts over time. Data were acquired at 16, 20, 24- and 27-days post-transduction and are presented as mean of three biological replicates (**E**) Confocal microscopy analysis of genetically modified DMD.A myoblast. DMD.A myoblasts derived from transduction experiments comprising AdV.mCherry.Fl-Dy, AdV.mCherry.Fl-Dys.TS, and AdV.Cas9.gRNA^{AAVS1} vectors were analyzed for detecting dystrophin, and β-dystroglican upon myoblasts differentiation. (WT: wild type).

References

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IMGENE- Improving Genome Editing Efficiency

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