CRISPR-Cpf1 correction of muscular dystrophy mutations in human cardiomyocytes and mice

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Introduction

• Duchenne muscular dystrophy (DMD) is an X-linked recessive disease caused by mutations in the gene coding for dystrophin

• dystrophin: large cytoskeletal protein essential for the integrity of muscle cell membranes

• DMD causes progressive muscle weakness, premature death by the age of 30 (cardiomyopathy)

• no effective treatment for this disease

• treatments: delivery of truncated dystrophin, utrophin by recombinant adeno-associated virus (AAV), skipping of mutant exons with antisense oligonucleotides and small molecules

Introduction

• these approaches cannot correct DMD mutations / permanently restore dystrophin expression

• new genome editing method as therapy?: CRISPR-Cas9 system (clustered regularly interspaced short palindromic repeats) for the correction of diverse genetic defects
  • adaptive immune system in bacteria that defends against phage infection
  • endonuclease (Cas9) guided to specific genomic sequences by guide RNA (gRNA) resulting in DNA cutting near a protospacer adjacent motif (PAM)
  • large size of SpCas9 reduces efficiency of packaging and delivery in AAV vectors
  • smaller CRISPR enzymes would facilitate precision gene editing
  • new RNA-guided endonuclease Cpf1 (CRISPR from Prevotella and Francisella 1)

https://www.addgene.org/crispr/guide/
Methods

• generation of pLbCpf1-2A-GFP and pAsCpf1-2A-GFP plasmids: LbCpf1 (*Lachnospiraceae bacterium ND2006*) and AsCpf1 (*Acidaminococcus sp. BV3L6*)

• human iPSCs from DMD patient fibroblasts (Riken HPS0164, deletion of exons 48 to 50, introducing a STOP codon within exon 51): nucleofection with plasmids, GFP+ and GFP- cells were sorted, single clones from GFP+ cells → sequencing, iPSCs induced to differentiate into cardiomyocytes (chemical induction)

• genomic DNA isolation (mouse fibroblasts, human DMD iPSCs)

• reverse transcription PCR (primers flanking DMD exons 47 and 52; amplified from WT cardiomyocytes, uncorrected cardiomyocytes and exon 51-skipped cardiomyocytes)

• dystrophin Western blot analysis

• dystrophin immunocytochemistry and immunohistochemistry
Methods

• mitochondrial DNA (mtDNA) copy number quantification (qPCR) → functional analysis of DMD iPSC-derived cardiomyocytes

• cellular respiration rates: oxygen consumption rates (OCR) determined in human iPSC-derived cardiomyocytes using XF24 Extracellular Flux Analyzer (Seahorse Bioscience)

• in vitro transcription of LbCpf1 mRNA and gRNA

• single-stranded oligodeoxynucleotide: ssODN used as a HDR template (template for correction of mutations)

• CRISPR-Cpf1-mediated genome editing by one-cell embryo injection in \textit{mdx} mice

• PCR amplification of genomic DNA

• T7E1 assay (mismatch-specific T7 endonuclease I assay)

• Tse I RFLP analysis (restriction fragment length polymorphism analysis)

• mouse forelimb grip strength test, serum CK measurement
Correction of DMD mutations by Cpf1-mediated genome editing

A. Reframing by INDELs and exon skipping.

B. Splice donor: Exon GT, Splice acceptor: Exon AG.

C. Target-DMD-Ex51 sequence:

5′-.. tatatttagCTCTACTCAGACTGTACTCTGGTG..-3′

3′-.. ataaaatcGAGGATGACTCTGACAATGAGAC..-5′

D. U6-gRNA CBh hCpf1 NLS 2A GFP pA

D. Transfection

E. GFP sorting assay:

- 293T cells
- Riken51 iPSCs (del 48-50)

LbCpf1 AsCpf1 LbCpf1 AsCpf1

GFP: - + - + - + - +

bp: 600 400 200 M
Results – Figure 2

DMD iPSC-derived cardiomyocytes express dystrophin after Cpf1-mediated genome editing by reframing

A

DMD-iPSCs → Editing → Corrected DMD iPSCs → Differentiation → Corrected cardiomyocytes

B

Uncorrected:

47 48 49 50 51 52 53

Reframed:

47 51 52

C

Exon 47 → Exon 51

Uncorrected:

AT AAA G C T C T A C T C A G A C I G T I C T I G I G I G A

Ile Lys Leu Leu Leu Arg Leu Leu Leu Trp Stop

Reframed:

AT AAA G C T C T A C T C A G A C I G T I C T I G I G I G A

Ile Lys Leu Leu Leu Arg Leu Val Thr Gln Pro
DMD iPSC-derived cardiomyocytes express dystrophin after Cpf1-mediated genome editing by reframing
Results – Figure 3

DMD iPSC-derived cardiomyocytes express dystrophin after Cpf1-mediated exon skipping.
Results – Figure 4

A. Dmd

B. g3, g2, g1

C. LbCpf1

D. Target-Dmd

Katrin Zlabinger
JC/TS Current Topics in Applied Immunology and Tissue Regeneration
SS2017
Results – Figure 5

CRISPR-LbCpf1–mediated *Dmd* correction in *mdx* mice

**A**

Donor mutant

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**B**

HDR-mediated correction

NHEJ-mediated reframing

In-frame deletion of premature stop codon

**C**

<table>
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<th>+T7E1</th>
<th>Litter 1</th>
<th>Litter 2</th>
<th>Litter 3</th>
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HDR (%) 16% 8% 50% 8% 25%
Results – Figure 5
CRISPR-LbCpf1–mediated Dmd correction in mdx mice

D

WT

mdx

mdx-C

E

WT

mdx

mdx-C

F

WT

mdx

mdx-C

H&E

TA

GFP

Dystrophin
Immunohistochemistry of skeletal muscles, heart, and brain from WT, *mdx*, and LbCpf1-edited mice (*mdx*-C)
Results – Supplement 7

H&E staining of skeletal muscles, heart, and brain from WT, *mdx*, and LbCpf1-edited mice (*mdx*-C)

<table>
<thead>
<tr>
<th>Muscle</th>
<th>WT</th>
<th><em>mdx</em></th>
<th>HDR-8%</th>
<th>HDR-25%</th>
<th>HDR-50%</th>
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<td>Heart</td>
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<tr>
<td>Brain</td>
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Western blot analysis of skeletal muscles, heart, and brain from WT, *mdx*, and LbCpf1-edited mice (*mdx*-C)
### Results – Table 1

**Table 1. Serum CK measurement and forelimb grip strength of WT, *mdx*, and LbCpf1-corrected *mdx*-C mice.** M, male; F, female.

<table>
<thead>
<tr>
<th>Mouse no.</th>
<th>Percent correction by HDR</th>
<th>Sex</th>
<th>Weight (g)</th>
<th>CK (U/liter)</th>
<th>Forelimb grip strength (grams of force)</th>
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<td>Trial 1</td>
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<td>M</td>
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<td>455</td>
<td>103</td>
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<td>M</td>
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<td>220</td>
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<td>M</td>
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<td>306</td>
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<td>F</td>
<td>15.5</td>
<td>184</td>
<td>86</td>
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<tr>
<td>WT-5</td>
<td>—</td>
<td>F</td>
<td>15.4</td>
<td>175</td>
<td>88</td>
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<tr>
<td>WT-6</td>
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<td>157</td>
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<td>F</td>
<td>16.2</td>
<td>4168</td>
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<td><em>mdx</em>-C1</td>
<td>16%</td>
<td>F</td>
<td>21.7</td>
<td>1233</td>
<td>112</td>
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<td><em>mdx</em>-C2</td>
<td>8%</td>
<td>F</td>
<td>19.7</td>
<td>4920</td>
<td>119</td>
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<td><em>mdx</em>-C3</td>
<td>50%</td>
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<tr>
<td><em>mdx</em>-C4</td>
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<td><em>mdx</em>-C5</td>
<td>25%</td>
<td>F</td>
<td>17.7</td>
<td>3239</td>
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</table>
Discussion

• newly discovered CRISPR-Cpf1 nuclease can efficiently correct DMD mutations in patient-derived iPSCs and \textit{mdx} mice, allowing for restauration of dystrophin expression

• robustness and efficiency of Cpf1 in mouse genome editing → HDR-mediated correction, ORF of mouse \textit{Dmd} gene was completely restored → fibrosis, inflammatory infiltration rescued

• two different strategies: reframing (only one gRNA needed) and exon skipping

• but differences in dystrophin expression level, mtDNA quantity, OCR in different edited clones → reframed dystrophin may not be structurally or functionally identical to WT dystrophin
Discussion

• use of one or two gRNA?
  • two are more effective for disruption of splice acceptor site
  • removes deleterious “AG” nucleotides → pseudosplice acceptor site generation eliminated
  • both gRNAs should cleave simultaneously which may not occur
  • one gRNA → uncertainty of length of INDELs?

• unique T-rich PAM sequence (G-rich PAM sequence in SpCas9)
• LbCpf1 is 140 AA smaller → enhances packaging and delivery by AAV
• LbCpf1 and AsCpf1 had high genome-wide targeting efficiency and high specificity comparable to those of SpCas9
• new powerful approach to permanently eliminate genetic mutations and rescue abnormalities associated with DMD and other disorders
Pro-Cons

• interesting new approach to eliminate genetic mutations, safe therapy? possible risks?
• iPSCs from just one patient? why not generating more iPSC clones from different patients?
• out of 24 pups born only 5 carried corrected alleles with different correction rates → just 8 to 50%, no 100% correction? and 50% only in one mouse
• for me personally difficult to read because of genetic engineering science vocabulary
Thank you for your attention!