Developing and testing nanoparticle encapsulated CRISPR-Cas9 plasmids for muscular dystrophy

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INTRODUCTION

- Duchenne muscular dystrophy (DMD) is a fatal, childhood disease affecting 1 in 5,000 males caused by mutations in the *DMD* gene, leading to loss of the dystrophin protein in muscle.[1]
- A potential treatment strategy for DMD is gene editing by clustered regularly interspaced short palindromic repeats (CRISPR) with the enzyme, caspase-9 (Cas9).[2]

HYPOTHESIS

CRISPR/Cas9 plasmids, when encapsulated with a liposomal nanoparticle, will improve gene editing and reduce off-target effects in the mouse model for DMD (mdx) when compared to non-encapsulated plasmids.

METHODS

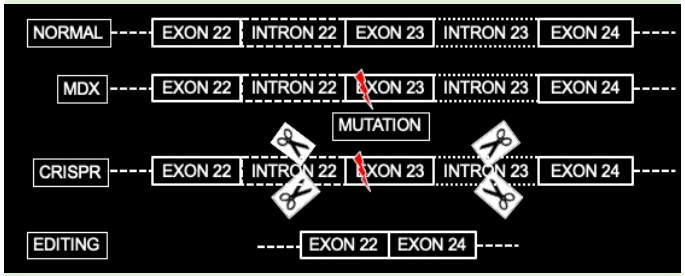


Figure 1: Normal DMD gene (top) in the region of exon 22 through exon 24. Mdx mouse DMD gene (second to top row) had a point mutation in exon 23 (red strike), leading to a stop codon and halting of dystrophin translation. We developed CRISPR sgRNAs (second from bottom row) 5' (intron 22) and 3' (intron 23) of exon 23 and the mutation. We also used previously published sgRNAs for comparison.[2] The sgRNAs and Cas9 would excise intron 22, exon 23, and intron 23, leading to a splicing of exon 22 and 24 together, resulting in a truncated, but functional dystrophin protein (editing, bottom row).

Single guide RNA development: We used Benchling software to develop sgRNAs surrounding the mutation in *DMD* exon 23 of the mouse genome. We developed 5' (intron 22) and 3' (intron 23) custom guides and also used a previously published 5' and 3' sgRNA.[2] Genecopoeia was assigned to develop our plasmids containing sgRNAs and Cas9.

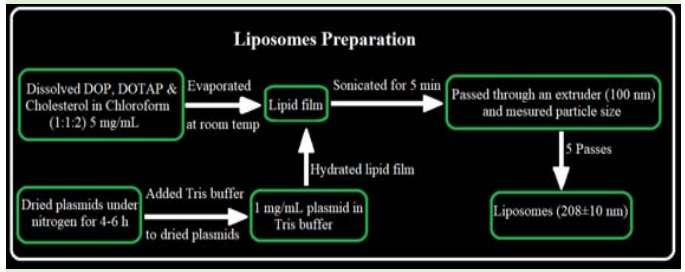
Plasmid creation, infection, transformation, and purification: Plasmid constructs were designed and generated by the investigators and Genecopoeia. SgRNA constructs contained CMV and T7 promoters and ampicillin and neomycin resistance genes. Glycerol stocks were grown in E. coli with ampicillin and DNA was purified according to the manufacturer's protocol (Endofree Plasmid Maxi Kit, Qiagen). Purified plasmid identity was confirmed via PCR and restriction digest (EcoRI, SbfI, AflII for 1h at 37˚C).

METHODS (contd.)

Table 1: CRISPR sgRNA development. We developed sgRNAs 1 and 2 and used previously published sgRNAs for comparison (A and B). BP = base pair length of plasmid (combined with Cas-9). MW = molecular weight in kiloDaltons.

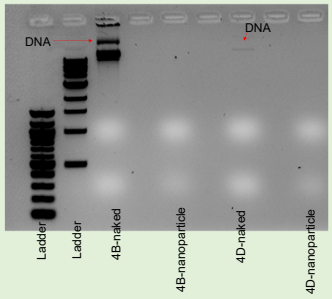
Guide ID	Source	Position	BP	MW (kDA)	Sequence	PAM
mdx-1	New Guide RNA Sequence	83803524 (intron 23)	10,839	7,045	CGAAAATTTCAG GTAAGCCG	AGG
mdx-2	New Guide RNA Sequence	83802871 (intron 22)	10,839	7,045	AGTATTTAATTTC CAGACTT	AGG
mdx-A	Long et al. 2016	83803137 (intron 22)	10,839	7,045	ATAATTTCTATTAT ATTACA	GGG
mdx-B	Long et al. 2016	83802393 (intron 23)	10,839	7,045	ATTTCAGGTAAGC CGAGGTT	TGG

Figure 2: Nanoparticle preparation and Encapsulation



RESULTS

Figure 3: We encapsulated various clones of the sgRNAs and ran an agarose gel electrophoresis on 4B and 4D clones. We were able to detect naked plasmid DNA in non-encapsulated plasmids, but not in encapsulated plasmid preps.



FUTURE EXPERIMENTS

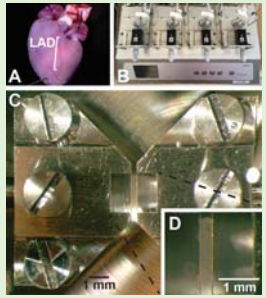


Figure 4: Examination of arterial function by wire myography. (A) 2 mm segments of the LAD are excised from the surrounding cardiac tissue, and functional responses are measured using a (B) Danish MyoTechnology (DMT) Model 620M wire myograph. Two 25 µm gold-plated tungsten wires are passed through the lumen of the LAD then transferred to the myograph chamber (C), which is filled with PSS and maintained at 37ºC. One wire is attached to a force transducer, and the other wire is attached to a micrometer driven support, which is used to apply isometric stretch to the vessel (D) comparable to the equivalent resting wall tension experienced in vivo at physiological pressures. After mice are treated with the encapsulated nanoparticle, arteries will be examined for functional changes.

CONCLUSION

- CRISPR/Cas9 has been used in the animal models for DMD,[3] but further studies are needed to improve safe delivery of plasmids.
- Further experiments are needed to isolate naked plasmid DNA and nanoparticle encapsulated plasmid to determine the relative amount of encapsulation. From here, we will perform in vivo studies with the optimal encapsulated plasmids and compared these to naked plasmid experiments.

ACKNOWLEDGEMENTS

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