Designing a CRISPR-Cas9 pipeline to investigate the effects of putative genetic modifiers on Duchenne Muscular Dystrophy (DMD)

Kamorah R. Ryhlick, Geremy T. Lerma, Natalie M. Aloi, Erin Miller, Joseph C. Beljan, Jared C. Talbot, and Sharon L. Amacher

The Department of Biology, The Ohio State University, Columbus, OH 43210

Abstract

Duchenne muscular dystrophy (DMD) is an X-linked neuromuscular disorder that affects about 1 in 3,500 live male births (Bushby et al. 2010). DMD is caused by a mutation of the Dystrophin (or DMD) gene that causes loss of dystrophin, a structural protein. Loss of dystrophin increases muscle's susceptibility to damage leading to muscle atrophy and loss of muscle function (Bass et al. 2016). Disease progression is variable in patients with DMD. Genome-wide association studies (GWAS) have identified putative genetic modifiers of DMD that may influence disease severity and variability (Bello et al. 2016; Flangian et al. 2013; Flanigan et al. 2021; Wess et al. 2018). I am using zebrafish, an established model organism to study DMD, to validate and further investigate these putative modifiers. CRISPR-Cas9 mutagenesis is highly efficient in zebrafish and can be used for rapid genetic screens by injecting Cas9 protein and a gene-specific sgRNA, targeting vital protein domains, to knock-out gene function and generate crispant fish. I designed sgRNAs to target zebrafish orthologs of DMD, LTBP4, THBS1, ETAA1, and PARD6G. Microinjection of zebrafish embryos with Cas9 and sgRNA targeting *dmd* causes loss of dystrophin protein, disorganization of muscle when assayed by birefringence, impaired motility, and decreased life-span of injected fish. To assay the effect of these genetic modifiers on *dmd*, multiplexed injections of *dmd* sgRNA + sgRNA targeting putative modifiers were carried out. To validate mutagenesis of putative modifiers hRMA was conducted after injection. Fish that show a *dmd* phenotype and mutagenesis of the putative modifier can then be used for subsequent analysis of muscle birefringence, motility, and lifespan. My data lays the foundational work to rapidly screen and identify modifiers of DMD for the development of new DMD therapeutics.

Background information

A genetic modifier is a gene which modifies the phenotypic outcome of the main gene that causes the disease.

- GWAS study identified LTBP4 and THBS1 as genetic modifiers of DMD (Flanigan KM, *et al.,* & Ann Neurol. 2018).
- DMD modifiers LTBP4 and THBS1 regulate transforming growth factor-β (TGFβ) bioavailability.
- Persistent TGFβ signaling drives fibrosis, persistent inflammation and inhibits muscle stem cell proliferation.
- Persistent TGFβ signaling drives DMD disease severity.

Project approach

Question:

Can CRISPR-Cas9 mutagenesis be used as a reliable and efficient system to generate F0 *dmd* cripsants? Using this system can we obtain double gene knockouts of *ltbp4* and *dmd* ?

Hypothesis:

CRISPR-Cas9 when paired with guide sgRNA will effectively target the desired gene to knock-out gene function.

Prediction:

When CRISPR-Cas9 and *dmd* sgRNA are microinjected into a zebrafish embryo at the one-cell stage a F0 *dmd* crispant will be obtained.

Method for designing a CRISPR-Cas9 guide pipeline

Method:

- Designed guides (sgRNAs) that target protein domains that are vital for protein function to generate a loss of function mutation.
- Microinject CRISPR-Cas9 and *dmd* sgRNA into zebrafish embryo at the one-cell stage.
- Microinject CRISPR-Cas9 and *dmd* + modifier sgRNA into embryo at the one-cell stage.
- Raised injected and uninjected zebrafish to 4 days post fertilization (4dpf).
- At 4 dpf zebrafish were fixed, and birefringence images were taken to assay *dmd* phenotype.
- At 4 dpf zebrafish performed free swimming assay to analyze swimming ability.

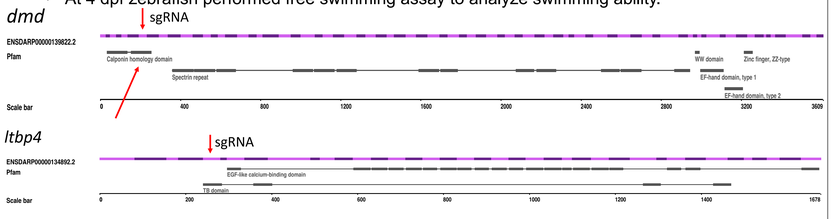

Figure 1: Designed guides targeting vital protein domains in *dmd* and *ltbp4* to knockout gene function. In the *dmd* gene two calponin homology domains were targeted. They make up an actin-binding domain which was hypothesized to be vital for protein function. In the *ltbp4* gene the TB domain was targeted. It is a vital TGFβ-binding domain and is known to sequester latent TGFβ.

CRISPR-Cas9 pipeline design

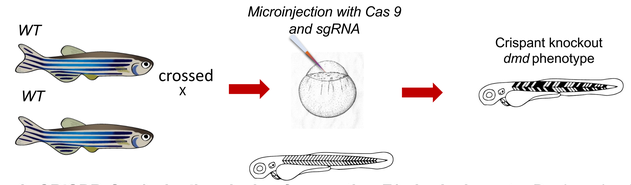

Figure 2: CRISPR-Cas9 pipeline design for creating F0 *dmd* crispants. Designed guides targeting protein domains to create a nonsense mutation. Microinjected CRISPR-Cas9 and *dmd* sgRNA into WT zebrafish embryo. Raised injected and uninjected zebrafish to 4 days post fertilization (4dpf). At 4 dpf the injected and uninjected zebrafish were fixed. Birefringence was used to assay *dmd* phenotype. The images were quantified using the system *ImageJ.*

F0 *dmd* crispants are similar to *dmd* mutants

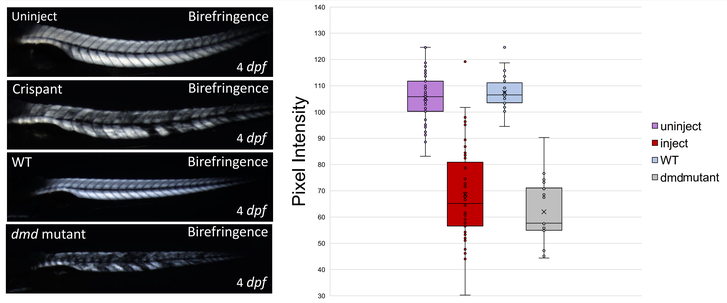

Figure 3: CRISPR-Cas9 mutagenesis is a reliable and efficient method to induce a *dmd* phenotype in comparison to *dmd* zebrafish mutants. Wild-type (WT) embryos were injected with a mix of CRISPR-Cas9 + *dmd* sgRNA. The injected, n=49, uninjected, n=62, WT, n=20, and *dmd* mutant, n=20, embryos were raised to 4 days post fertilization (dpf). Birefringence images were taken of the injected, uninjected, WT, and *dmd* mutant zebrafish at 4 dfp (left) and quantified for pixel intensity (right). Welch's t-test (pvalue=0.083) shows a nonsignificant difference in pixel intensity between injected and *dmd* mutant. A *dmd* crispant phenotype was observed via birefringence with an efficiency of 0.82 in injected zebrafish. Injected embryos that did not display a *dmd* crispant phenotype were omitted from the data set.

dmd;ltpb4 multiplex injections did not rescue *dmd* birefringence phenotype

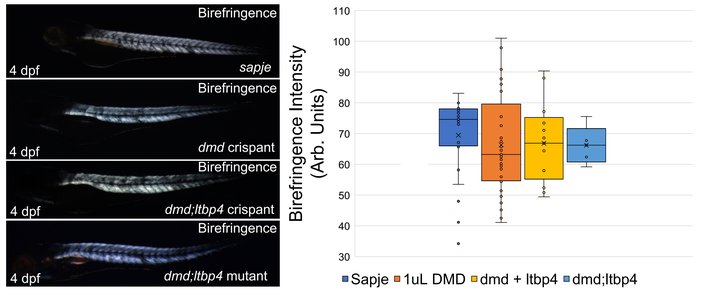

Figure 4: *dmd;ltbp4* multiplex injections resulted in mutagenesis at targeted gene locations. *dmd* mutagenesis was screened using birefringence (left) and *ltbp4* mutagenesis was validated via hRMA. *Sapje dmd* mutants, *dmd;ltpb4* mutants, and *dmd* crispants were used as controls to compare *dmd;ltbp4* crispants. Welch's t-test was performed between *dmd+ltbp4* to *sapje* (pvalue 0.595); *dmd+ltbp4* to 1uL *dmd* (pvalue: 0.912); *dmd+ltbp4* to 0.5uL *dmd* (pvalue: 0.125); *dmd+ltbp4* to *dmd;ltbp4* mutants (pvalue: 0.91). This data reveals that *dmd;ltpb4* multiplex injections did not rescue *dmd* birefringence phenotype (right). *Figure provided by graduate student Geremy Lerma, Amacher lab.*

Assaying motility data between dmd and WT zebrafish

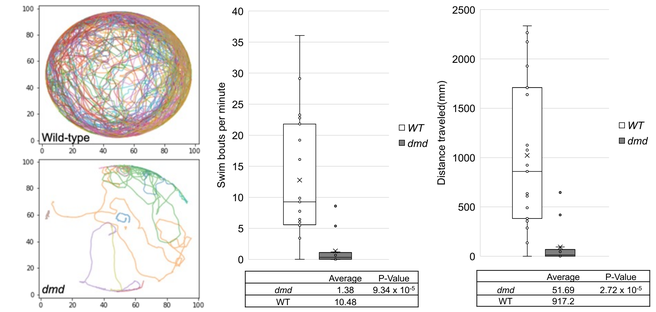

Figure 5: Wild-type fish exhibit more swim bouts than *dmd* mutant fish. Free swimming assays demonstrate global differences in movement patterns (left) as well as differences in total movement activity (right). Welch's t-test identifies a significant decrease in activity in *dmd* mutant embryos (p-value: 9.34 x 10^{-5}) and distance traveled (p-value 2.72 x 10^{-5}). Nine additional movement parameters can be extracted from these assays. *Figure provided by graduate student Geremy Lerma, Amacher lab.*

Summary

- Guides were designed targeting vital protein domains in the *dmd* and *ltbp4* gene (Figure 1).
- Microinjection with CRISPR-Cas9 and *dmd* sgRNA resulted in *dmd* F0 crispants that displayed a muscle phenotypes that was not significantly different from *dmd* mutants (Figure 3).
- Multiplex injections of *dmd;ltbp4* did not rescue *dmd* birefringence phenotype (Figure 4).
- Analyzation of the locomotion of *dmd* zebrafish in comparison to WT zebrafish reveals that the swimming ability of *dmd* zebrafish is significantly decreased (Figure 5).
- This data has helped validate and establish a CRISPR-Cas9 pipeline that can efficiently and quickly generate *dmd* F0 crispants.

Together, these data demonstrate that our approach is a promising one to efficiently validate the effects of genetic modifier loss on *dmd*.

Future directions

I anticipate that my future work will evaluate the impact and influence of putative genetic modifiers, LTBP4, THBS1, PARD6G, and ETAA1, on muscle birefringence, motility, and lifespan of *dmd* mutants.

- Guides have been designed for each modifier of interest, and microinjections of CRISPR-Cas9 and *dmd* sgRNA + modifier sgRNA are currently underway.
- To validate the mutagenesis of putative modifiers, I will use high-resolution melting analysis to detect the mutations and to determine guide efficiency after injection.
- Subsequent analysis of muscle birefringence, motility, and lifespan will indicate whether modifier gene loss affects any aspect of the *dmd* phenotype.

References

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