Assessing anti-dystrophin T-cell responses by ELISpot following AAV9-microdystrophin gene therapy in dogs

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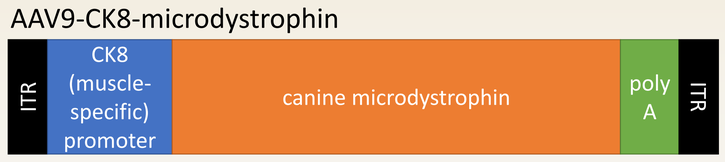
Introduction

Duchenne muscular dystrophy (DMD), which is caused by mutations in the X-linked dystrophin gene, affects approximately one in 3,500 to 5,000 newborn males. It is differentiated from the milder Becker muscular dystrophy by a complete or near-complete absence of the dystrophin protein, often the result of unstable mRNA due to early stop codons and frame-shifting mutations. A recent focus of treatment for DMD has been the use of adeno-associated viral vector (AAV) mediated gene transfer and subsequent expression of a miniaturized form of dystrophin (microdystrophin) in muscle to reduce disease severity. To assess anti-transgene immunological responses, we conducted a pre-clinical assessment of T-cell responses to canine microdystrophin following administration of a novel AAV9 vector cassette, which utilizes the muscle-specific promoter CK8, in DMD dog colonies at two different sites.

AAV9-CK8-microdystrophin



Experimental design

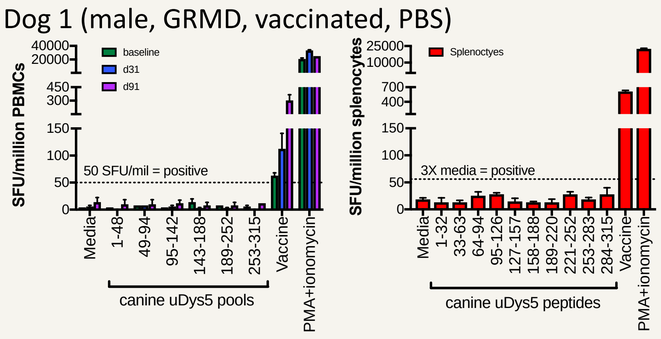
The first dog colony used was the golden retriever-derived model (GRMD), with a point mutation at the 3' splice site of intron 6 resulting in skipping of exon 7, a frame shift and an early stop codon in exon 8. The second colony was a mixed-mutation colony, with the GRMD mutation, the Pembroke Welsh corgi insertion of LINE-1 in intron 13 resulting in a novel exon with an early stop codon, and the Labrador retriever insertion of 184 base pairs in intron 19, resulting in a nonsense mutation.

Dogs received doses between 1e13 and 5e14 vg/kg, or vehicle control, delivered systemically at 2-4 months of age with transient immune suppression and were followed for at least 3 months. Peripheral blood mononuclear cells (PBMCs) were collected at baseline and 2 timepoints post AAV-administration. Splenocytes were collected from dogs from the first colony at necropsy. PBMCs and splenocytes were then used in IFNγ ELISpots in which cells were stimulated with PMA + ionomycin (positive control), media alone (negative control), or canine microdystrophin peptides (15mers with 11 amino acid overlap). Additionally, the combination vaccine (parvovirus, adenovirus, influenza, distemper) administered during routine vaccination was tested as a potential physiologic positive control. Samples were run in duplicate and were considered positive if the number of spots was 3X background and >50 Spot Forming Units (SFU)/million cells.

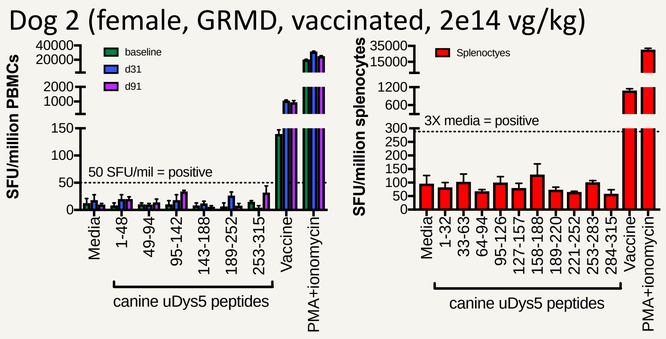
Results

Colony 1: GRMD

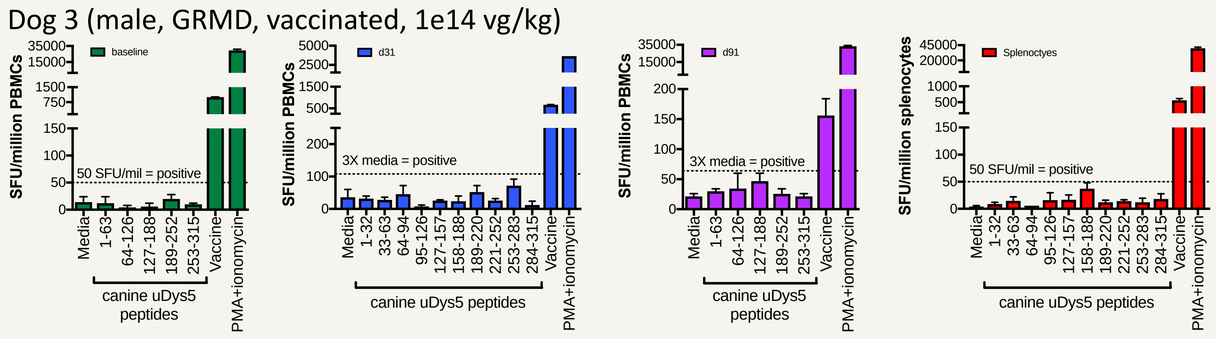
Dog 1 (male, GRMD, vaccinated, PBS)



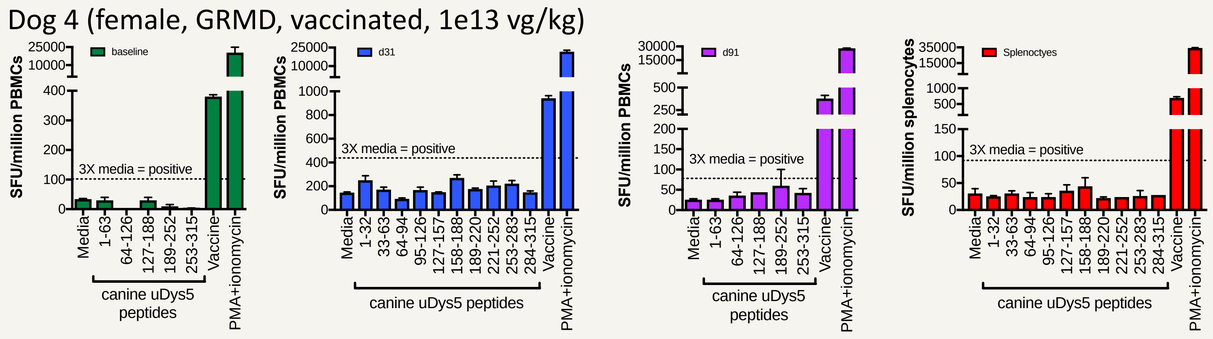
Dog 2 (female, GRMD, vaccinated, 2e14 vg/kg)



Dog 3 (male, GRMD, vaccinated, 1e14 vg/kg)

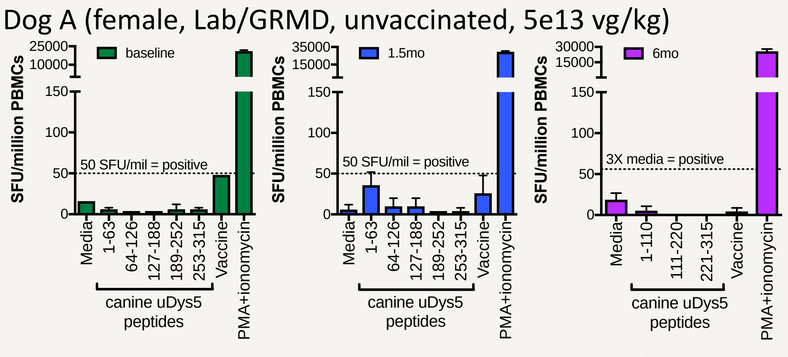


Dog 4 (female, GRMD, vaccinated, 1e13 vg/kg)

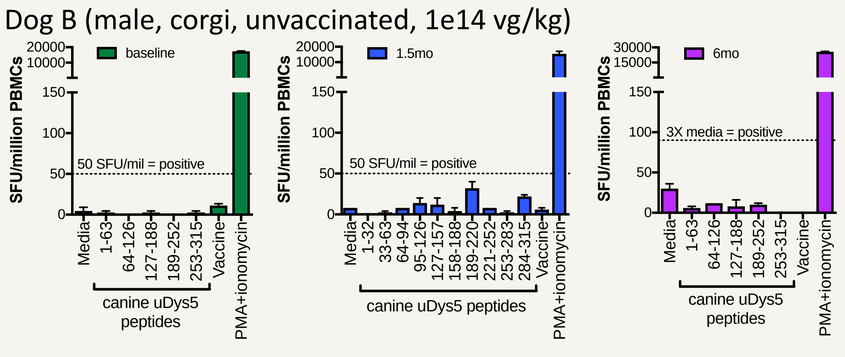


Colony 2: GRMD, Pembroke Welsh corgi, Labrador retriever

Dog A (female, Lab/GRMD, unvaccinated, 5e13 vg/kg)

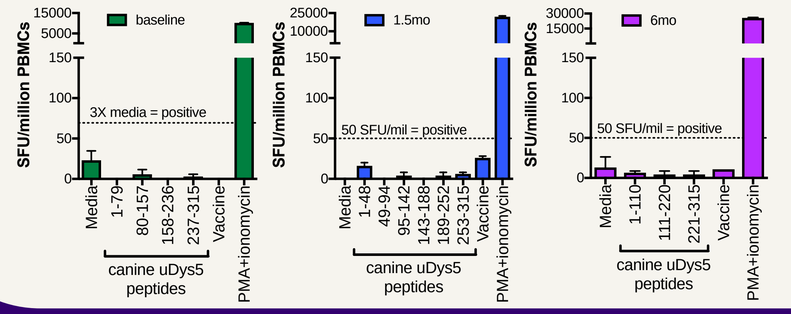


Dog B (male, corgi, unvaccinated, 1e14 vg/kg)

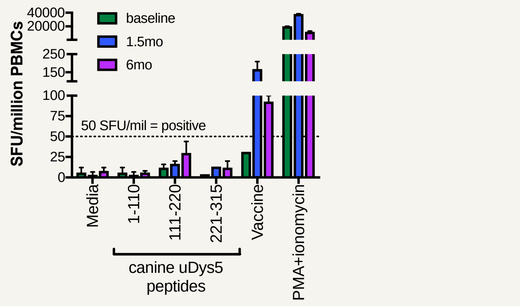


All graphs: Mean spot forming units (SFU) of duplicate wells are plotted with standard deviation error bars.

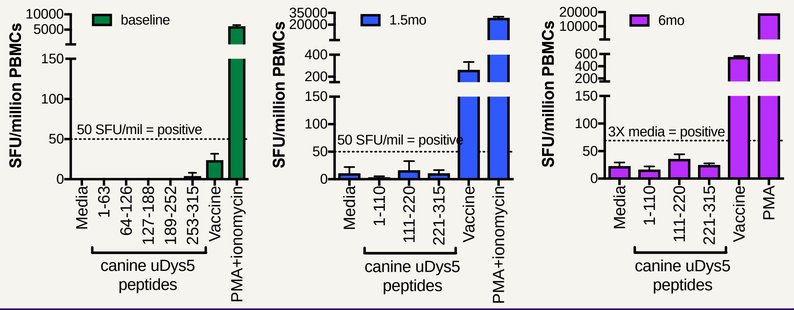
Dog C (female, GRMD/corgi, unvaccinated, 1e14 vg/kg)



Dog D (male, Lab, vaccinated, 3e14 vg/kg)



Dog E (male, Lab, vaccinated, 5e14 vg/kg)



Conclusions

- The combination vaccine (parvovirus, adenovirus, influenza, distemper) administered during routine vaccination is an effective physiologic positive control.
- 8 dogs treated with AAV9-CK8-microdystrophin showed no evidence of anti-dystrophin T-cell responses
  - 2 colonies
  - Three different mutations
  - Doses ranging from 1e13 to 5e14 vg/kg
  - Up to six months of follow-up

Funding

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Disclosures for Lead Author

JMC owns Solid Biosciences stock and serves as a consultant for AskBio. Note this is a change from the time of abstract submission.

In Silico Platform for the Design and Generation of Novel Muscle Promoters: *In Vitro* Validation

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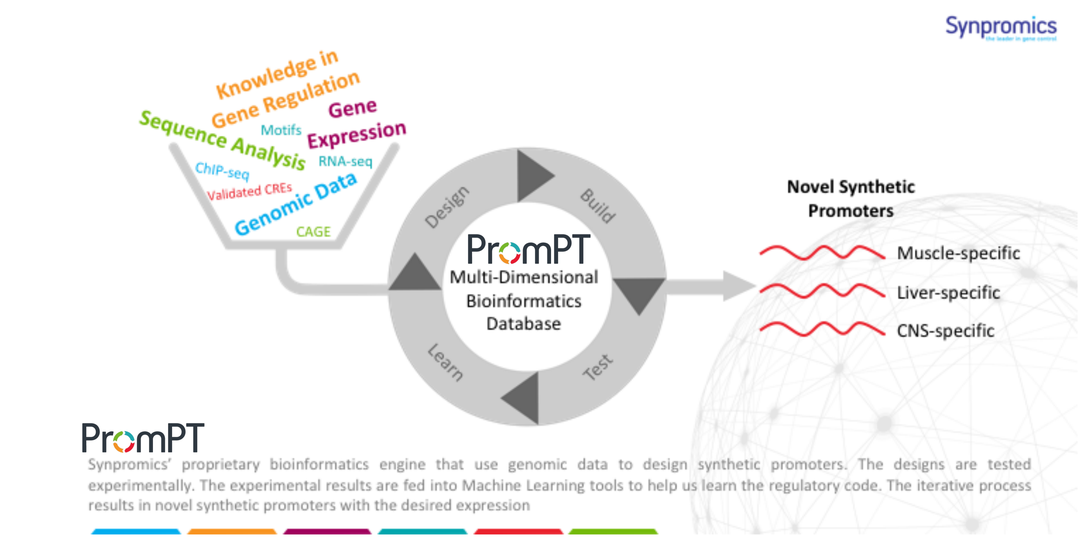
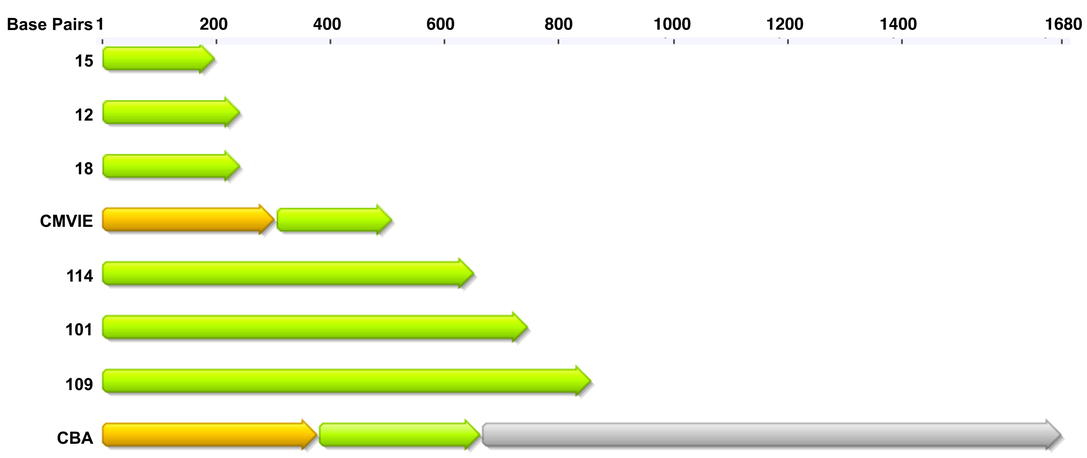
Abstract

Gene therapy-mediated transgene activity is driven by specialized DNA sequences that direct protein expression in either a ubiquitous or tissue-specific fashion. These sequences, termed promoters, can be derived from either naturally occurring endogenous sequences or synthetically designed to accommodate certain parameters such as tissue specificity, viral packaging limitations or to drive varied levels of protein expression. In this study, a panel of *in silico* designed synthetic promoters were generated and assessed for their ability to drive high levels of skeletal muscle-specific protein expression. Bioinformatics analysis of multiple genomic datasets (including gene expression and epigenetics data) was used to identify candidate enhancer and core promoter regions active in a set of genes differentially over-expressed in muscle. The bioinformatics analysis was done using PromPT®, a proprietary bioinformatics platform optimized for the identification of genomic regulatory regions. Candidate enhancer elements were synthesized upstream of the core promoter and transfected into skeletal muscle cells. Promoter activity was assessed in differentiated myotubes. Enhancer elements were then combined to create novel muscle specific synthetic promoters. Multiple candidate synthetic promoters were shown to have higher activity than CMVIE, a ubiquitous promoter, in differentiated rodent and human skeletal muscle cells. Importantly, these promoters demonstrated high tissue specificity, with minimal expression in non-muscle cell types, including kidney and liver cells. These data suggest that entirely novel regulatory sequences can be derived from computational analyses and tailor-suited to specific pre-designated requirements.

Background



Promoter Identification



- **Bioinformatics analysis was used to identify enhancer regions present in a set of genes differentially over-expressed in the muscle**
- **Candidate enhancer elements were synthesized upstream of core promoter and transfected into a variety of muscle cell types**
- **Enhancer elements were then combined to create novel muscle promoters**
- **Novel promoters ranged in sizes from approximately 200 base pairs to 850 base pairs (green arrow: promoter, orange arrow: enhancer, grey arrow: intron)**

Results

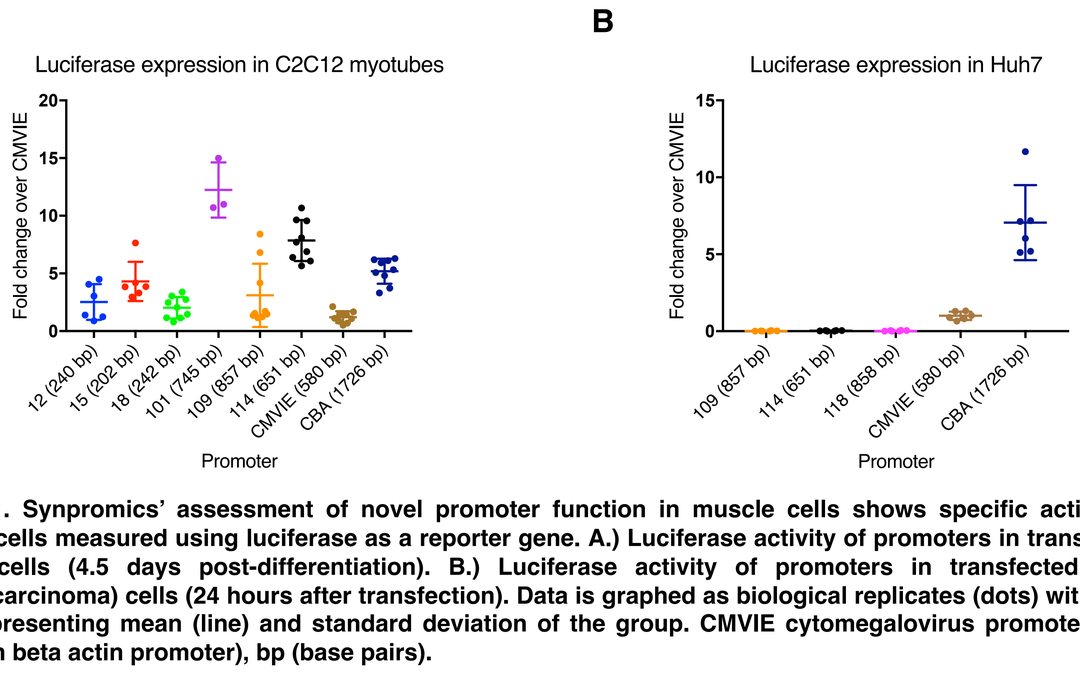
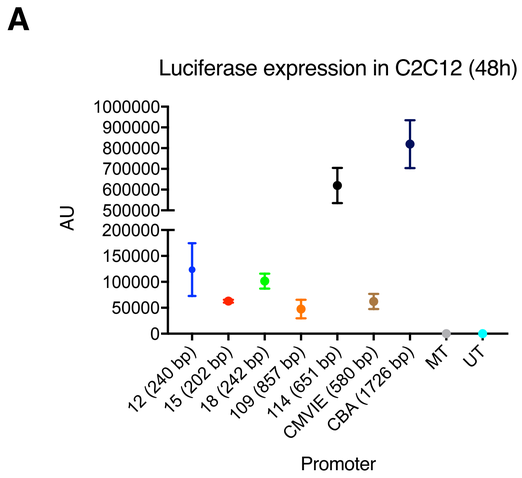
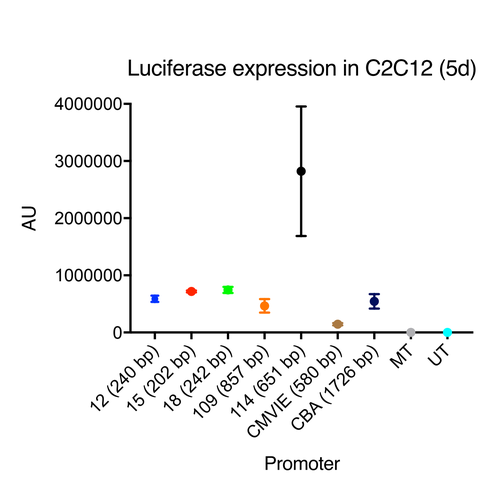
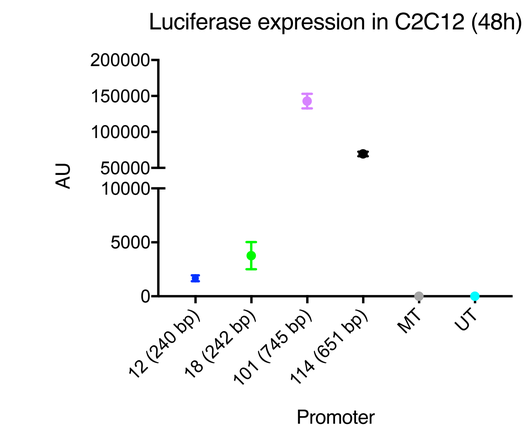


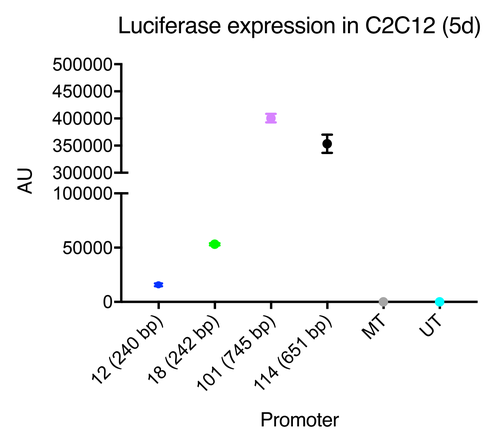
Figure 1. Synpromics' assessment of novel promoter function in muscle cells shows specific activity in muscle cells measured using luciferase as a reporter gene. A.) Luciferase activity of promoters in transfected C2C12 cells (4.5 days post-differentiation). B.) Luciferase activity of promoters in transfected Huh7 (hepatocarcinoma) cells (24 hours after transfection). Data is graphed as biological replicates (dots) with error bars representing mean (line) and standard deviation of the group. CMVIE cytomegalovirus promoter, CBA (Chicken beta actin promoter), bp (base pairs).

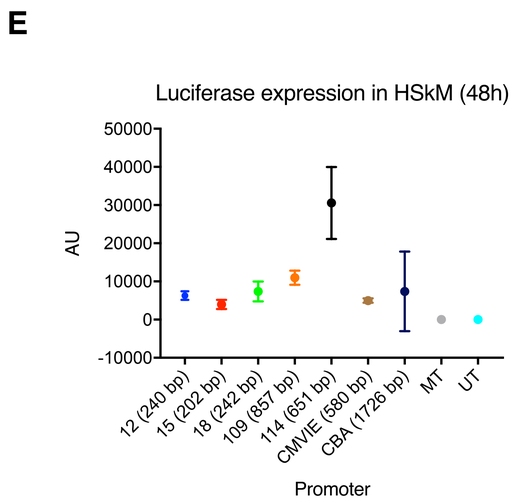
Results

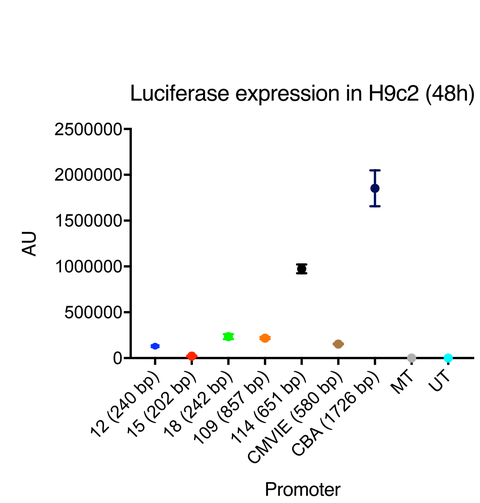












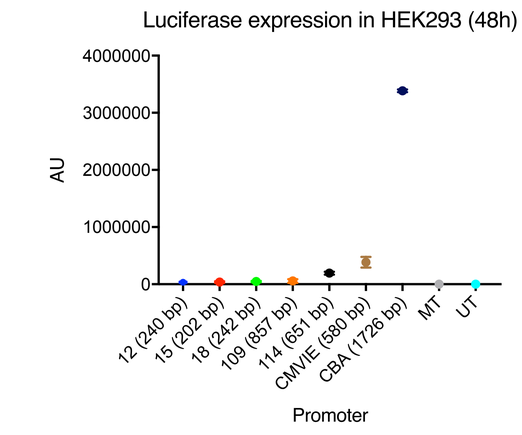


Figure 2. Solid's validation of novel promoters' activity and specificity by luciferase expression in muscle and non-muscle cells. Luciferase activity of promoters in transfected C2C12 cells A., C.) 48 hours and B., D.) 5 days post differentiation. E.) Human skeletal muscle (HSkM) cells 48 hours. F.) H9c2 rat cardiomyocytes 48 hours. G.) Human embryonic kidney (HEK) cells 48 hours. Data is graphed as mean and standard deviation of experimental replicates. AU (arbitrary units), MT (mock transfected), UT (untreated), CMVIE cytomegalovirus promoter, CBA (Chicken beta actin promoter), bp (base pairs).

Conclusion

- **The *in silico* designed novel promoters evaluated are specifically active in both skeletal and cardiac muscle cells.**
- **A range of expression was seen in both mouse and human myoblasts, with all promoters expressing higher than CMVIE promoter.**
- **Strong sustained transgene expression was observed in mouse myotubes with Synpromics promoters.**
- **The sizes and range of expression of these novel muscle specific promoters are amenable to viral mediated gene therapy for muscle diseases.**

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Preclinical Evaluation of SGT-001 Microdystrophin Gene Transfer for Duchenne Muscular Dystrophy

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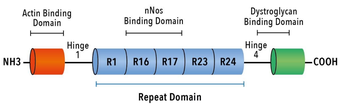
Solid Biosciences Inc., Cambridge, MA, USA

Introduction

Duchenne Muscular Dystrophy

- Duchenne muscular dystrophy (DMD) is a fatal neuromuscular disease caused by mutations in the *DMD* gene that lead to the absence of functional dystrophin protein
- Dystrophin stabilizes the dystrophin glycoprotein complex (DGC) at the sarcolemma and maintains local nitric oxide (NO) production by anchoring neuronal nitric oxide synthase (nNOS) to the DGC
- Without dystrophin, DGC members and nNOS lose sarcolemmal localization and show decreased overall protein levels and function
- As a result, muscles become susceptible to contraction-induced injury and functional ischemia, and break down over time
- Although the cause of DMD is well known, the largest challenges to developing a therapy are the size of the *DMD* gene (considered the largest protein-encoding gene in the human genome) and the need to deliver a therapy systemically to all muscles in the body

SGT-001



- SGT-001 is a recombinant adeno-associated virus serotype 9 (rAAV9) vector containing a microdystrophin transgene under the control of the muscle-specific CK8 promoter
- The microdystrophin transgene in SGT-001 maintains critical elements for dystrophin function, including the nNOS binding domain, while still fitting within AAV packaging limits
- Canine SGT-001 contains a canine-codon optimized microdystrophin
- SGT-001 is administered systemically by intravenous (IV) delivery to produce microdystrophin protein in skeletal and cardiac muscle

Scalable Manufacturing

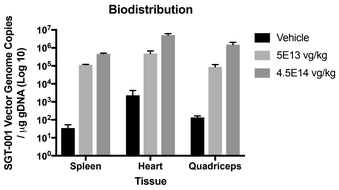
- SGT-001 manufacturing methodology evolved from an adherent cell-based method of research-grade production to a suspension culture method of clinical-grade production for increased scalability
- Suspension culture allows for production runs of hundreds to thousands of liters, which is essential to treat all patients amenable to therapy at potentially efficacious vector genome (vg)/kg dose levels

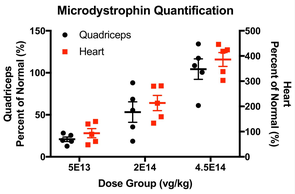
Preclinical Package

- Preclinical safety studies have been completed that show SGT-001 is well-tolerated at target dose levels in small and large animals
- Preclinical efficacy studies in small and large animal models of DMD show that a single IV dose of SGT-001 produces widespread, durable expression of microdystrophin in muscle tissues, with associated improvements in muscle histopathology and functional measurements

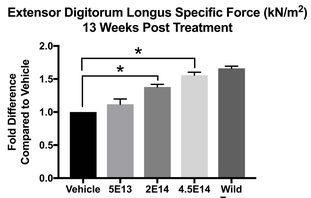
Results

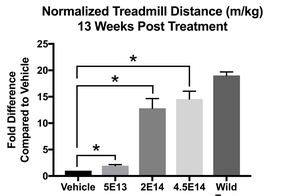
SGT-001 Treated mdx Mice Show High Levels of Microdystrophin Expression and Rescued Muscle Function After 3 Months





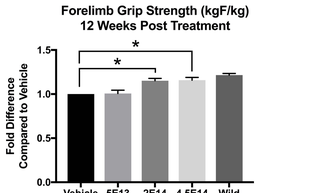
Data represent mean ± SEM | Animal numbers per dose group: n=5

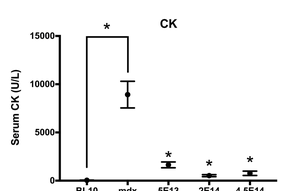




Data represent mean ± SEM | Animal numbers per dose group: Vehicle n=12, 5E13 n=10, 2E14 n=11, 4.5E14 n=10, Wild Type n=12 | * p<0.05

Data represent mean ± SEM | Animal numbers per dose group: Vehicle n=12, 5E13 n=11, 2E14 n=12, 4.5E14 n=12, Wild Type n=12 | * p<0.05

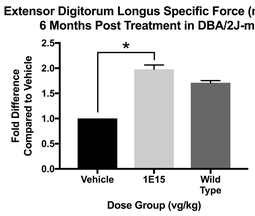


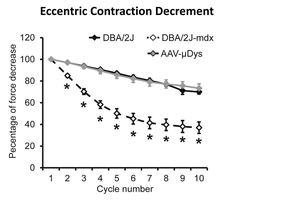


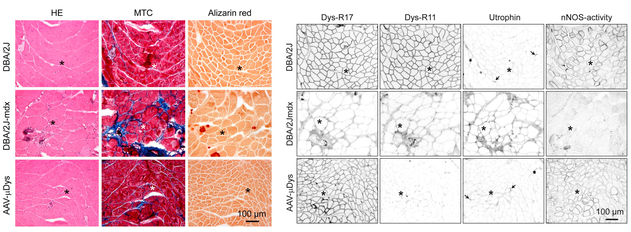
Data represent mean ± SEM | Animal numbers per dose group: Vehicle n=12, 5E13 n=11, 2E14 n=12, 4.5E14 n=12, Wild Type n=12 | * p<0.05

Data represent mean ± SEM | Animal numbers per dose group: Vehicle n=12, 5E13 n=11, 2E14 n=12, 4.5E14 n=12, Wild Type n=12 | * p<0.05

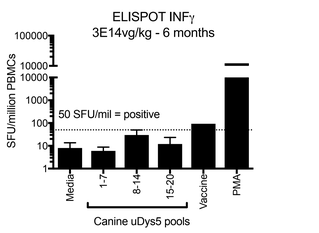
DBA/2J-mdx Mice Treated with Canine SGT-001 Show Sarcolemmal Microdystrophin Expression, nNOS Activity and Muscle Function Near WT Levels After 6 Months

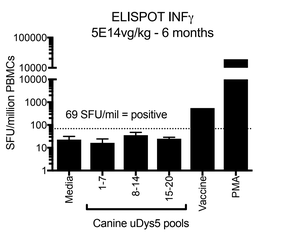




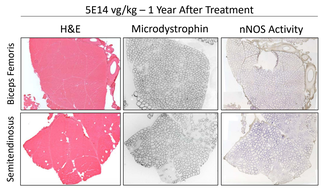


At Doses Up To 5E14 vg/kg of Canine SGT-001, Canine Models of DMD Show No ELISPOT Response to Microdystrophin

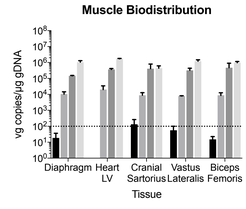


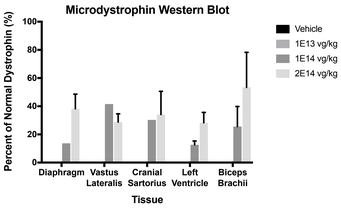


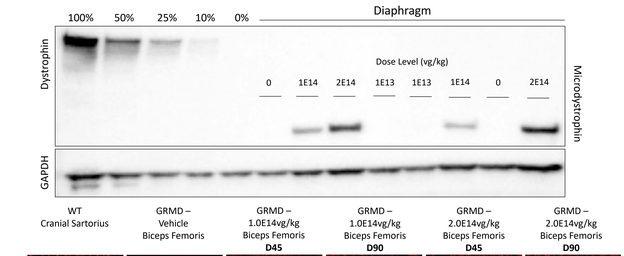
Microdystrophin Expression Persists for Multiple Years After a Single Systemic Administration of Canine SGT-001 in cDMD Dogs

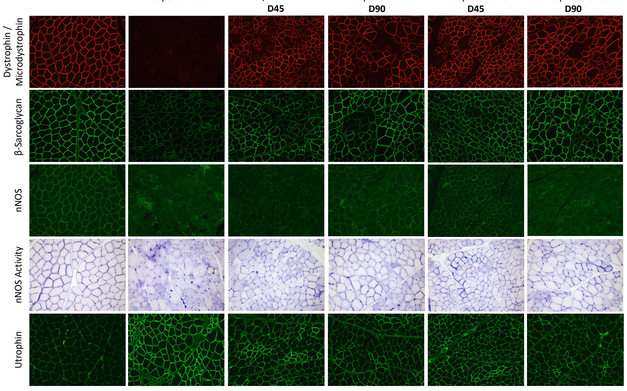


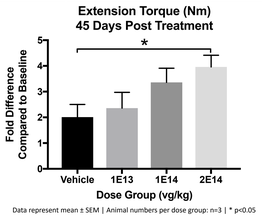
GRMD Canine Models of DMD Treated with Canine SGT-001 Display Bodywide Microdystrophin Expression in Muscle, Restored Expression of Dystrophin Associated Proteins and Significantly Improved Muscle Function



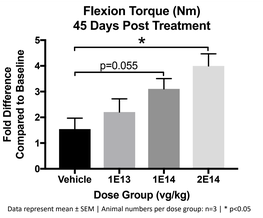








Data represent mean ± SEM | Animal numbers per dose group: n=3 | * p<0.05



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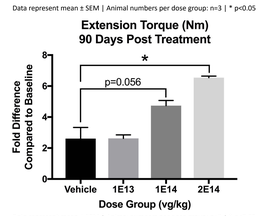

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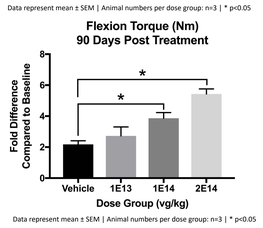

Data represent mean ± SEM | Animal numbers per dose group: n=3 | * p<0.05

Conclusions

- SGT-001 systemic administration results in widespread microdystrophin expression across target muscle tissues in a dose-dependent manner
- Microdystrophin expressed by SGT-001 shows molecular functional efficacy, reestablishing the dystrophin glycoprotein complex at the sarcolemma and importantly resulting in active nNOS
- Microdystrophin expression corresponds with improvements in both overall muscle histopathology and muscle function in small and large animal models of DMD
- SGT-001 mediated microdystrophin expression is durable, and persists for multiple years after administration of canine SGT-001
- Canine models of DMD treated with canine SGT-001 do not show signs of an immune response to microdystrophin by ELISPOT analysis
- Data suggest SGT-001 may be a suitable candidate for DMD therapy

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Complementary Techniques to Evaluate Microdystrophin Expression in Duchenne Muscular Dystrophy Gene Therapy Studies

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Duchenne Muscular Dystrophy

- Duchenne muscular dystrophy (DMD) is a fatal neuromuscular degenerative disease resulting from mutations in the *DMD* gene leading to the absence of functional dystrophin protein.
- Dystrophin stabilizes the dystrophin glycoprotein complex (DGC), which dissipates contractile forces out of myofibers thereby protecting them from contraction-induced injury

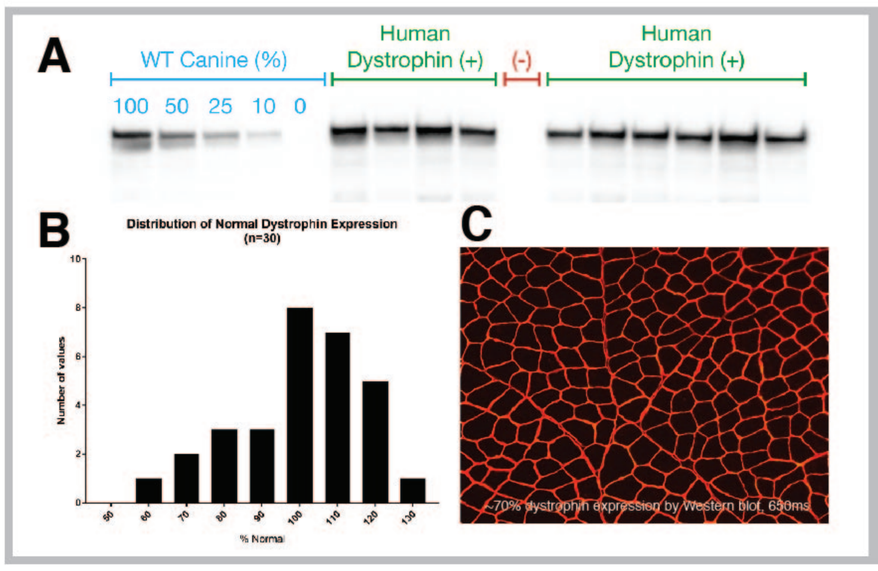


Figure 1: Range and distribution of normal human dystrophin expression

(**A, B**) Dystrophin protein expression in clinically and histologically normal skeletal muscle biopsies varies from 56.7% to 126.6% (n=30)

(**C**) Quantitative Western blots can accurately define this range, while traditional immunohistochemistry staining can saturate at high expression levels and 100% myofiber positivity is not 100% dystrophin expression

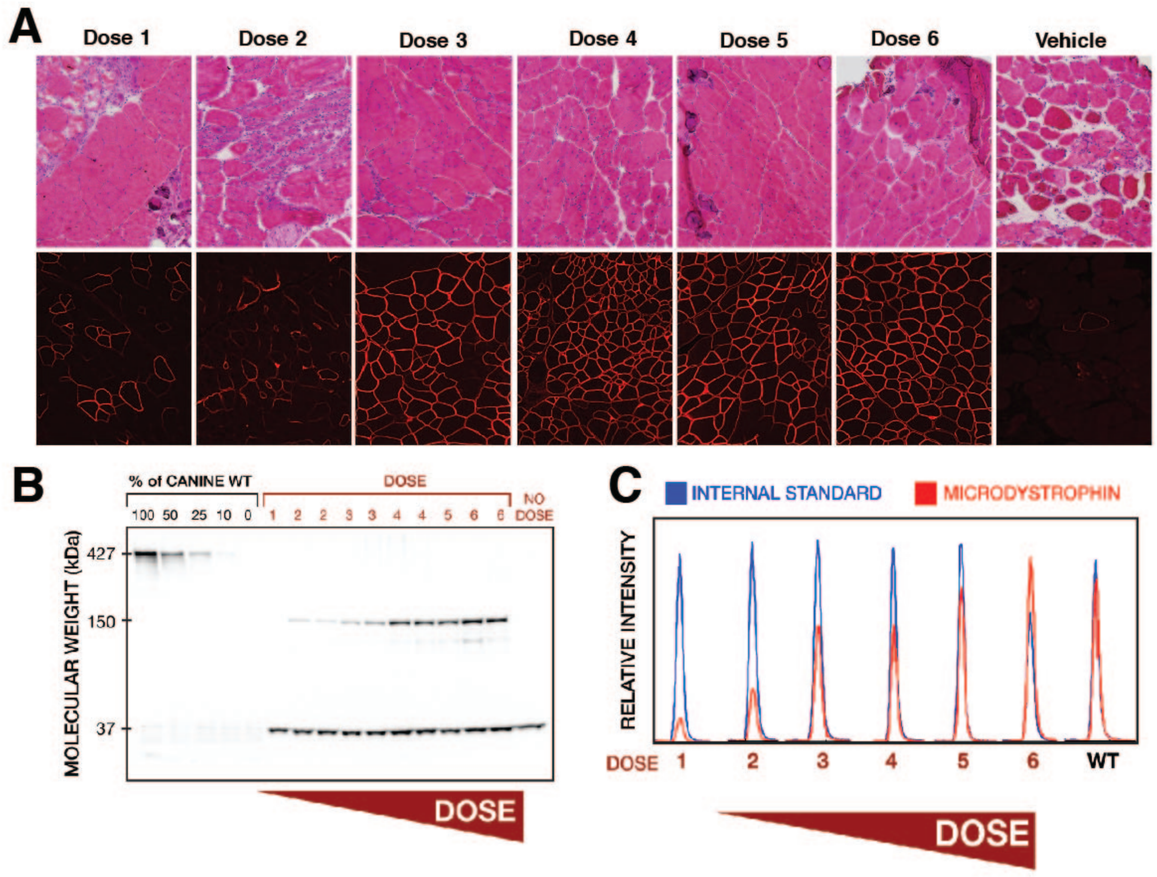


Figure 2: Value of complementary techniques in assessing microdystrophin levels in the context of a SGT-001 dose escalation study
SGT-001 was injected into 1 month old mice at 6 different doses and tissues were collected 28 days later
(**A**) Increasing doses of SGT-001 produce increasing levels of microdystrophin. Greater levels of microdystrophin expression in the quadriceps muscles are generally associated with less myofiber degeneration on H&E stained slides. Immunofluorescence staining also demonstrates a dose response
(**B**) Western blots utilizing a wild-type (WT) canine curve to quantify expression of microdystrophin in treated mouse hearts, illustrating a dose-dependent increase in microdystrophin expression
(**C**) Mass spectrometry data reveals a similar dose-dependent increase in microdystrophin expression

B

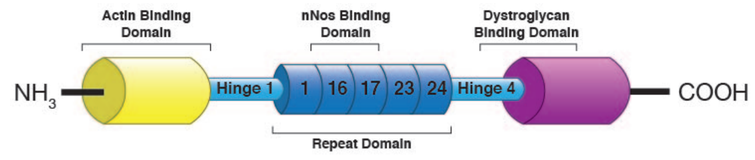
Step	Considerations
Calibration curve standards	Control muscle, diluted with dystrophic tissue
Protein extraction from tissue	Different buffers affect solubility and storage
Extracted protein quantification	Assay type, buffer, standard curve protein all affect values
Gel selection and running	Most labs use commercial, precast gels and standard running conditions
Membrane	PVDF, Nitrocellulose and a variety of voltage, times and temperatures
Blocking	Traditional milk, BSA or vendor proprietary mixes
Secondary antibody	Complementary to primary, concentrations tested
Detection	ECL or vendor proprietary options
Imaging	Imaging system or traditional film
Quantification	Based on calibration curve regression
Loading control	Dual detection, separate detection, muscle specific, use for correction or just QC
Pass/fail criteria	Curve linearity, loading control specificity, lowest band over zero densitometry

C

Step	Considerations
Tissue preparation	Tissue collection, freezing, or fixation • For muscle tissue, freezing, must be performed using isopentane
Species	Antibody binding and background signal can vary by species
Blocking agents	Different blocking agents can improve signal/noise. This may depend on antibodies and species under study
Detection method	Enzyme or fluorescent labels, colocalization, stability/fading, cost, intensity • Photography and documentation must reflect equivalent magnification and exposures.
Interpretation	Controls, non-specific background, lab to lab variation
Quantification	Manual vs. automated, % positive fibers vs. overall signal intensity

Figure 4: Technical considerations in assessments of dystrophin expression
(**A**) Different human primary antibodies have different affinities for mouse dystrophin due to lack of sequence homology
(**B**) A variety of factors should be considered when developing western blot techniques for the detection of dystrophin and all optimized and standardized
(**C**) Similarly, detection of dystrophin by immunofluorescence may be affected by a variety of factors

SGT-001

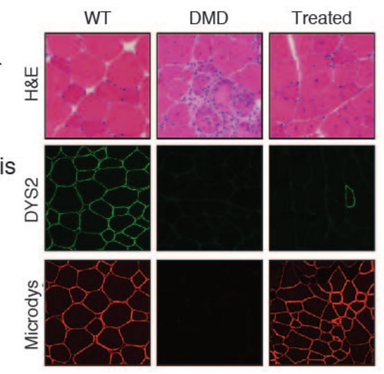


- SGT-001 utilizes the adeno-associated virus serotype 9 (rAAV9) containing a microdystrophin transgene and the muscle-specific CK8 promoter
- SGT-001 is delivered systemically by the intravenous (IV) route of administration to produce microdystrophin protein in both skeletal and cardiac muscles

Methods

Histology

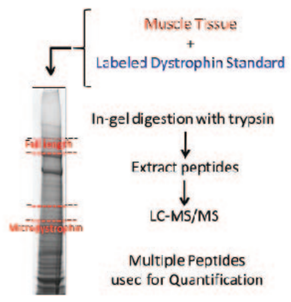
Isopentane-frozen muscle tissues are evaluated by H&E and immunofluorescence (IF) methods. IF is used to identify localization of microdystrophin (microdys), full length dystrophin (DYS2), nNOS, and β-Sarcoglycan. nNOS activity is assessed using histochemical staining. Percent of dystrophin positive fibers is scored by a neuropathologist. DYS2 enables identification of revertant fibers in mdx mice, GRMD dogs, and DMD humans separate from microdystrophin expression.



Full Length vs. Microdystrophin Expression

Western Blot

Muscle proteins are extracted, separated by SDS-Page, transferred to a membrane and probed with an antibody reactive to full length and microdystrophin proteins.



Mass Spectrometry Diagram

Mass Spectrometry

Muscle proteins are extracted, spiked with a stable isotope labeled standard, and separated by SDS-Page. The region corresponding to dystrophin and/or microdystrophin migration is excised, in-gel digested and analyzed by targeted LC-MS/MS. Ratios between standard and endogenous peptides are used for quantification.

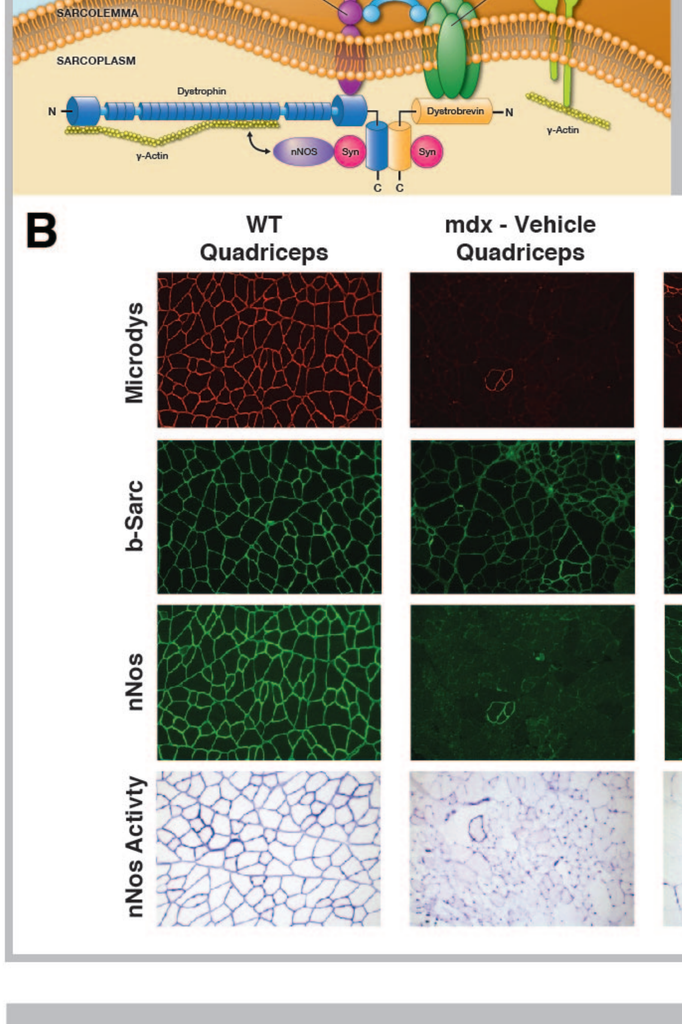


Figure 3: Dystrophin associated glycoprotein expression as an indicator of microdystrophin function.

(**A**) Diagram of the interactions between dystrophin and key dystrophin associated proteins.

(**B**) DMD models display decreases in beta sarcoglycan (b-Sarc) and nNOS compared to controls. Treatment with SGT-001 shows dose-dependent restoration of these proteins to the sarcolemmal membrane.

Discussion

- Both quantitative and qualitative assays are useful for characterization of dystrophin and microdystrophin expression
- Immunofluorescence studies are sensitive to low levels of dystrophin expression, demonstrate membrane localization and secondary markers are supportive of microdystrophin function
- We have developed a strategy using multiple techniques and markers to assess treatment-related expression of microdystrophin after treatment with SGT-001
- Dose-escalation studies using SGT-001 in mice displayed dose-dependent increases in microdystrophin expression by immunofluorescence, western blot, and mass spectrometry techniques. Appropriate function of microdystrophin was indicated by the restoration of secondary markers at the sarcolemmal surface, including beta sarcoglycan and nNOS
- Optimization of dystrophin detection will depend on the agent and species under study. A variety of factors can contribute to signal/noise issues and signals can be greatly improved with experimentation.