# Utility of a 3D Engineered Skeletal Muscle Organoid System to Assess Exon Skipping and Dystrophin Protein Restoration in a Human DMD Cell Model

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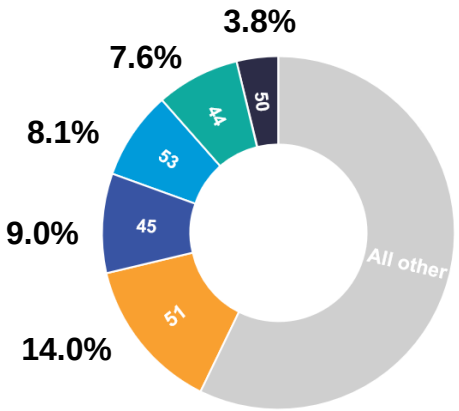
# DUCHENNE MUSCULAR DYSTROPHY (DMD)

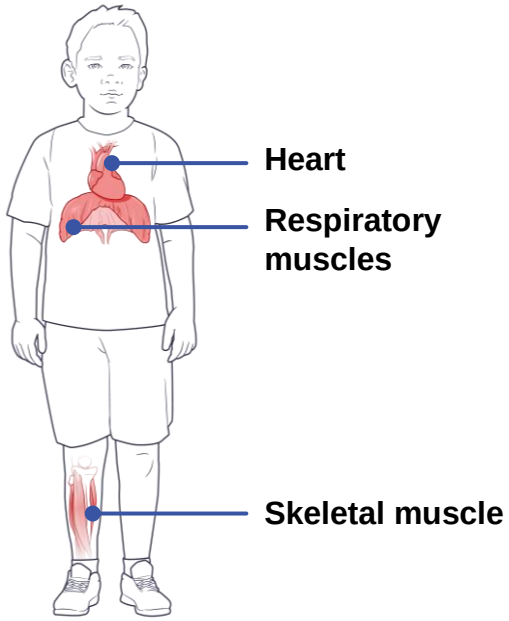
Duchenne is caused by **mutations in the *DMD* gene, which lead to a lack of functional dystrophin**, causing progressive loss of muscle function throughout the body

Exon skipping therapeutics have been approved based on **modest improvement in dystrophin levels ranging from ~1% to 6%**[4-7]

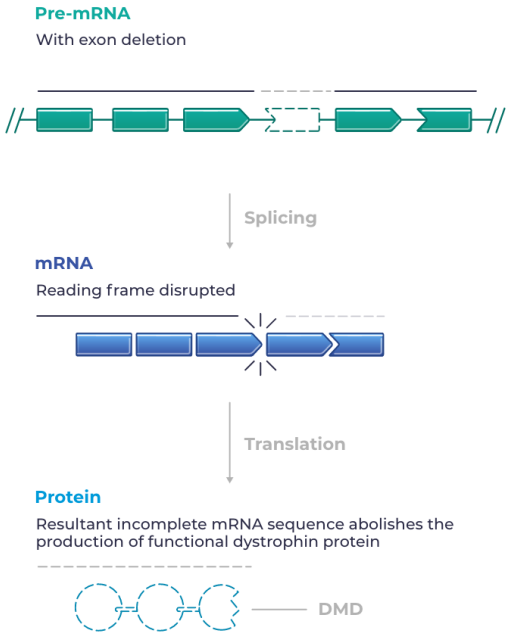
**~15,000** people in the **U.S.**[1] **&** **~26,000** people in **Europe**[2] have Duchenne

**>40% of patients with Duchenne**[3] have mutations amenable to exon skipping of exons 44, 45, 50, 51 and 53

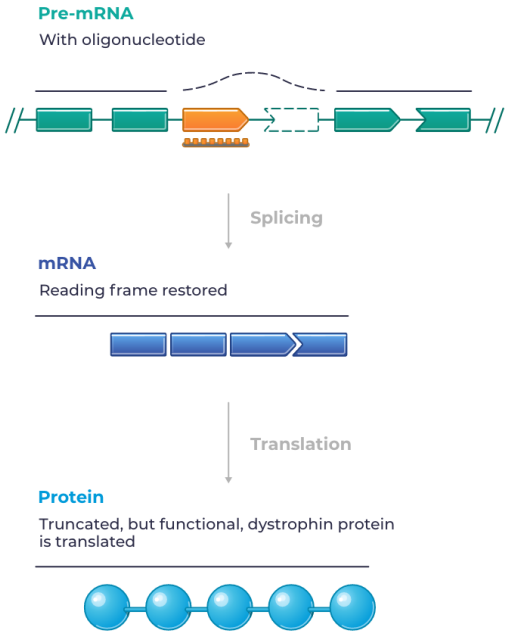



## Patients with Duchenne




## Exon Skipping Approach



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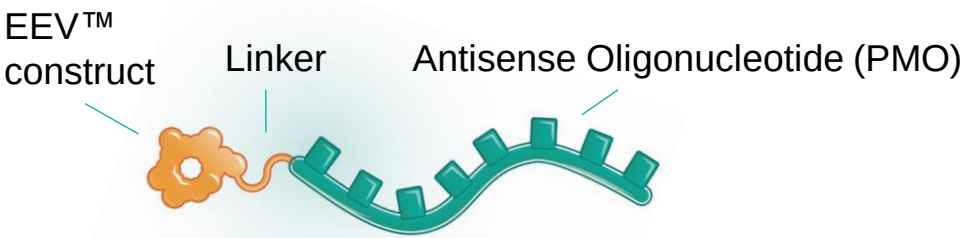
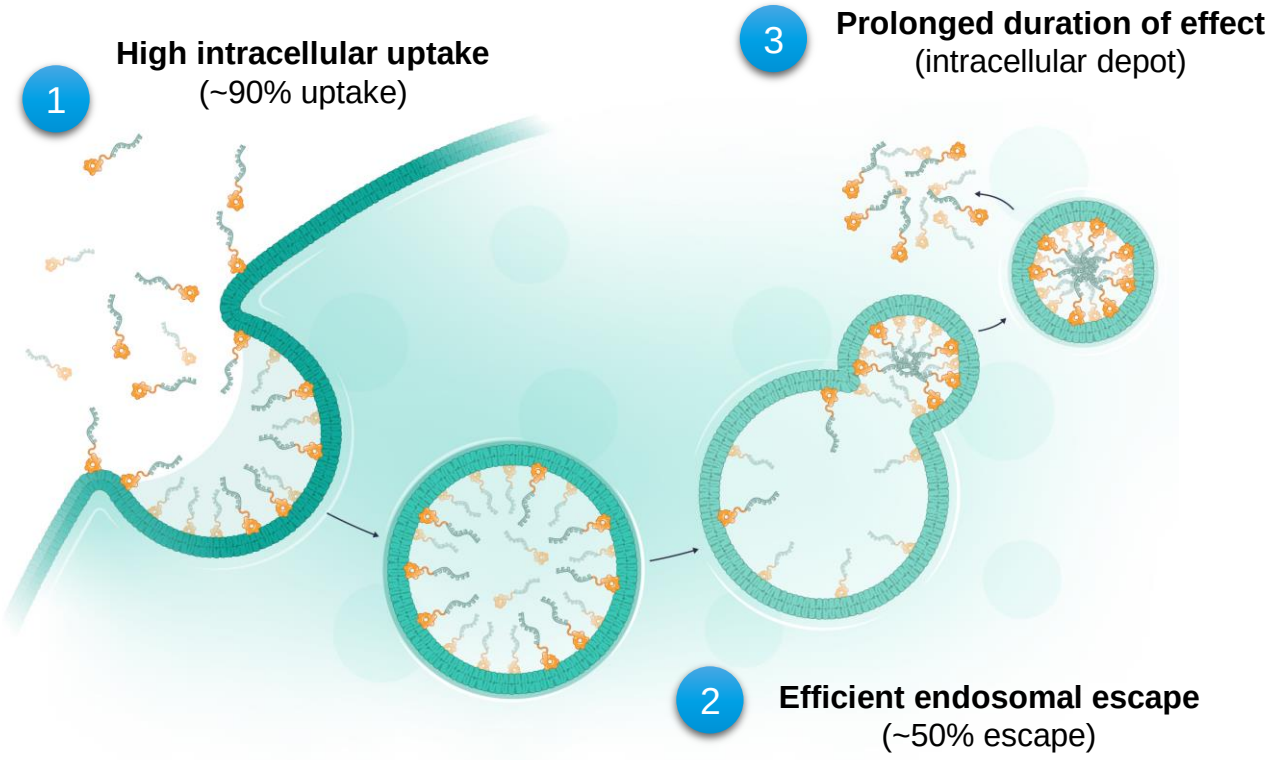
**1.** Parent Project Muscular Dystrophy. https://www.parentprojectmd.org/about-duchenne/. Accessed August 18, 2023. **2.** Europeans Medicines Agency. https://www.ema.europa.eu/en/medicines/human/orphan-designations/eu3202375. Accessed August 18, 2023. **3.** Bladen, C.L. et al. *Hum Mutat*. 2015. **4.** AMONDYS 45 PI. **5.** VILTEPSO PI. **6.** VYONDYS 53 PI. **7.** EXONDYS 51 PI. **DMD**, Duchenne muscular dystrophy; **EEV**, endosomal escape vehicle

# ENDOSOMAL ESCAPE VEHICLE (EEV™) PLATFORM

## The EEV Platform was developed to enhance the intracellular delivery of therapeutic cargo such as antisense oligonucleotides




- Unique chemistry results in **improved uptake and endosomal escape**
- Cyclic structure designed to **extend half life and increase stability**
- Phospholipid binding potentially **enables broad biodistribution to all cells**
- Mechanism of **internalization conserved across species**



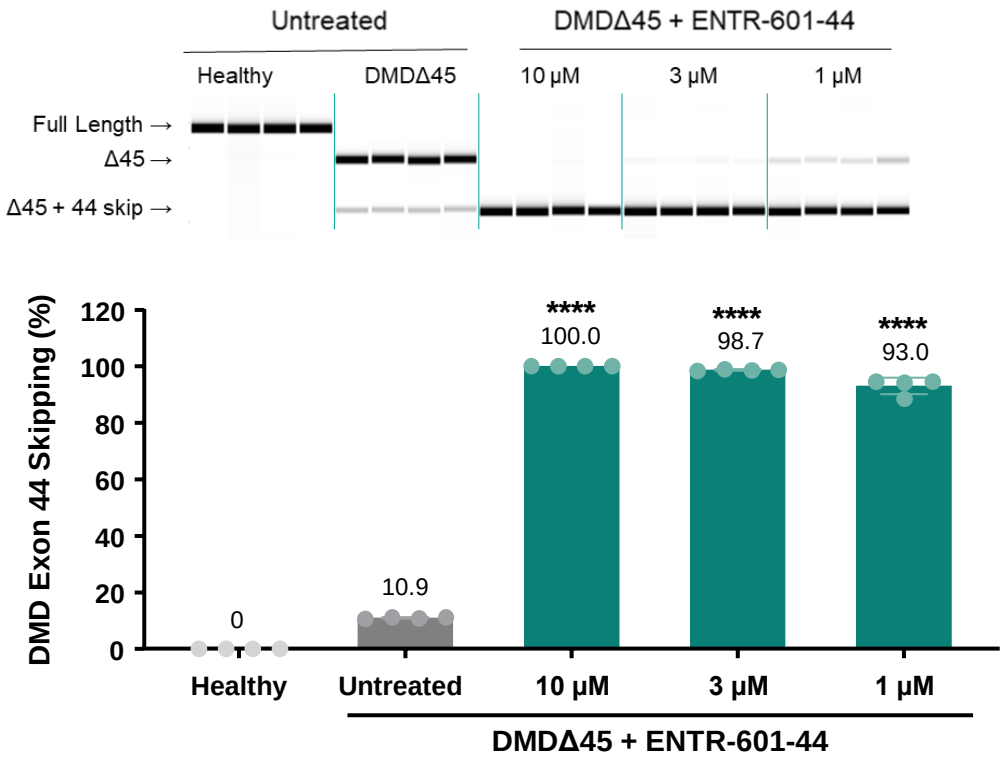



Qian, Z. et al. *ACS Chem. Biol.* 2013; Qian, Z. et al. *Biochemistry* 2014; Qian, Z. et al. *Biochemistry* 2016; Sahni, A. et al. *ACS Chem. Biol.* 2020; Pei, D. *Acc. Chem. Res.* 2022. **EEV**, endosomal escape vehicle. **PMO**, phosphorodiamidate morpholino oligomer.

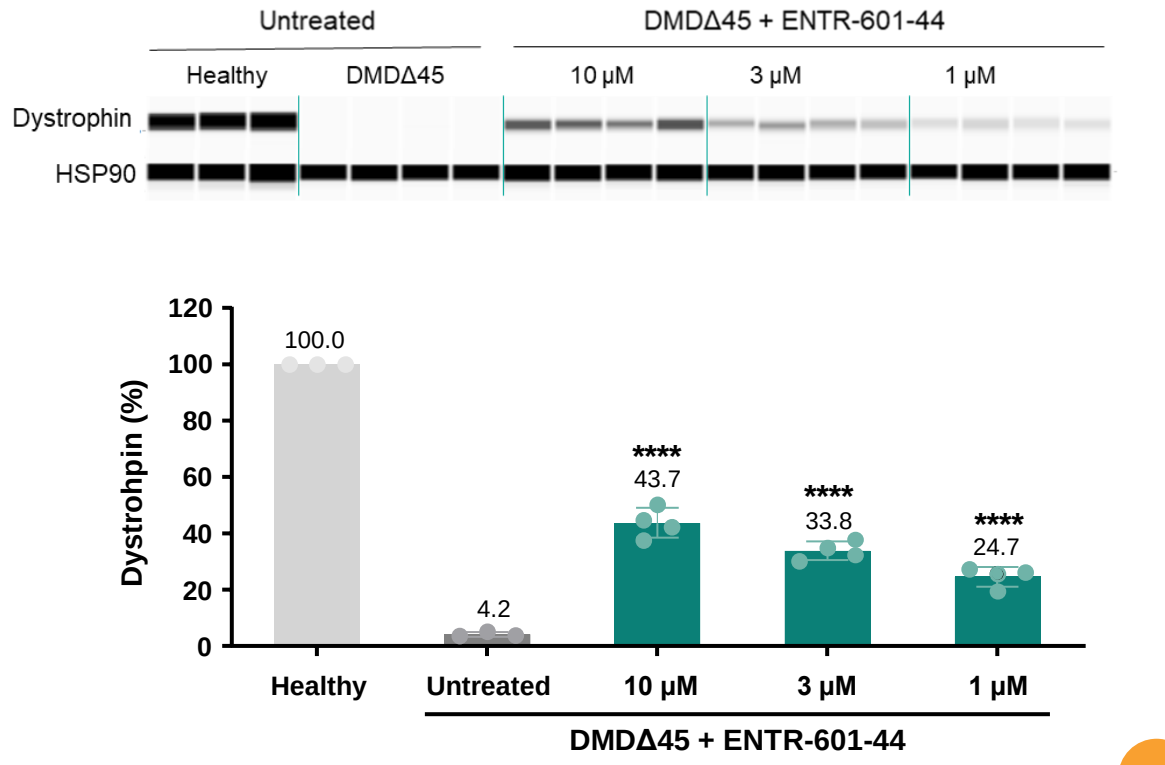
# EEV-PMO IN 2D PATIENT-DERIVED MYOTUBES

## Robust dose-dependent exon 44 skipping and dystrophin protein production were observed in DMD patient-derived muscle cells treated with an exon 44 skipping PMO

### Exon Skipping




### Dystrophin Protein Production



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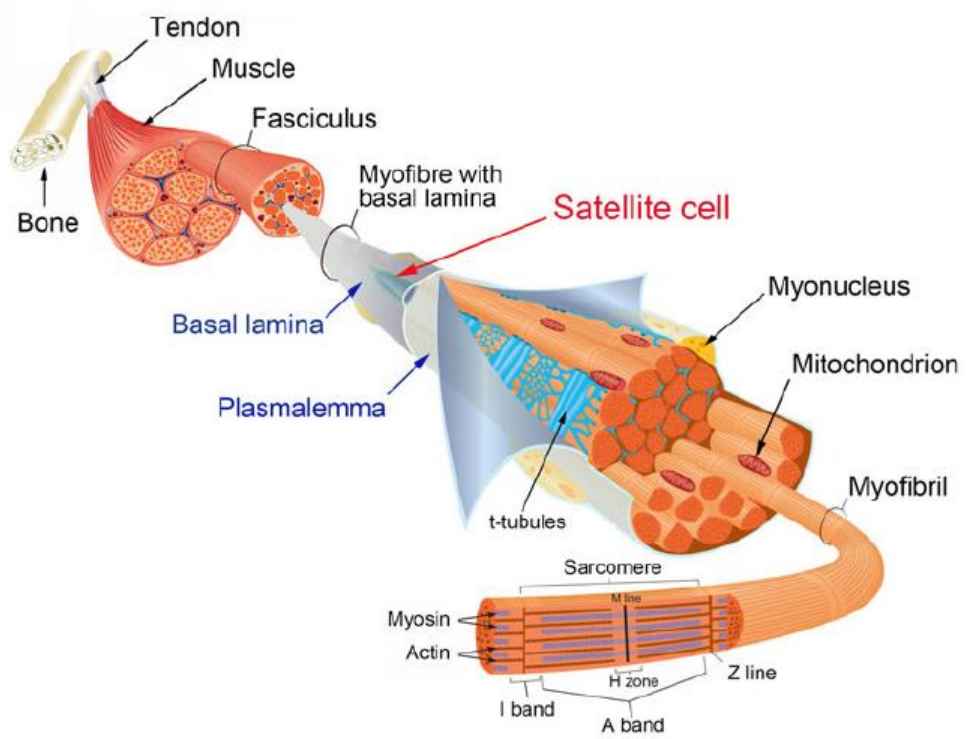
****p<0.0001 vs untreated DMDΔ45; Values shown as mean ± standard deviation. **DMDΔ45** are immortalized myoblasts from DMD patients harboring an out-of-frame exon 45 deletion and further differentiated into myotubes. **ENTR-601-44** is a DMD exon 44 skipping EEV-oligonucleotide conjugate. **DMD**, Duchenne muscular dystrophy; **EEV**, endosomal escape vehicle; **HSP90,** heat shock protein 90.

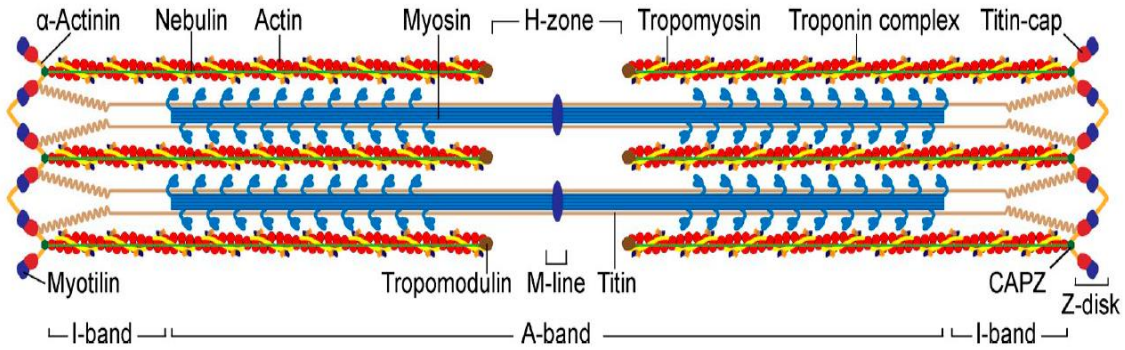
# SKELETAL MUSCLE MATURATION IS LIMITED WITH TRADITIONAL 2D CULTURE METHODS

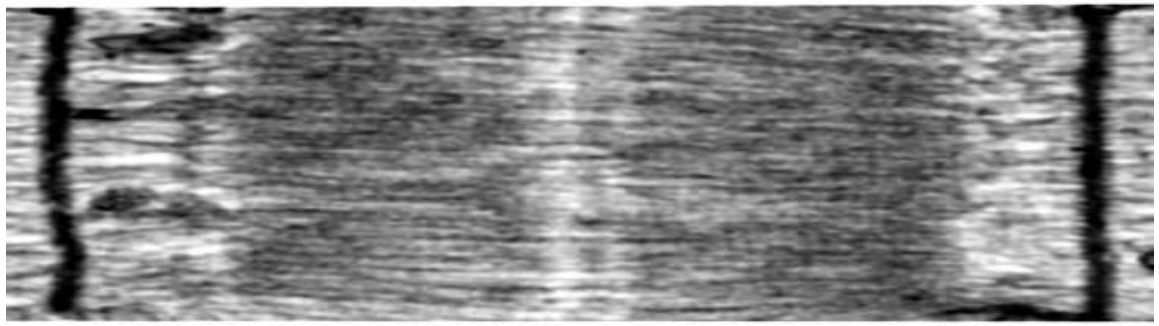
## Skeletal Muscle Structure[1]




- Skeletal muscle structure is complex and highly structured
- The sarcomere is basic contractile unit of striated muscle, and poorly defined with traditional culture methods

## Sarcomere in Skeletal Muscle Section[2,3]



- Proper sarcomere organization is not achievable with traditional cell culture methods in 2D

1. Relaix, F. & Zammit, P.S. *Development* 2012. 2. Rahimov, F. & Kunkel, L.M. *JCB* 2014. 3. Rassier, D.E. *Am J Physol Cell Physiol* 2017.

# ADVANTAGES OF 3D TISSUE CULTURE FOR MODELING NEUROMUSCULAR DISORDERS

Skeletal muscle maturation and culture longevity is limited with conventional 2D methods

## Challenges with traditional 2D culture systems

- Absence of scaffolding produces non-linear myotubes with random structural organization.
- Uncontrolled fusion results in undesirable levels of variability between experiments
- Culture maturation is typically limited to 4-10 days due to myotube detachment caused by spontaneous contractility.
- Gene expression changes in response to drug exposure can take weeks to months.
- Clinically relevant functional measurement not feasible.

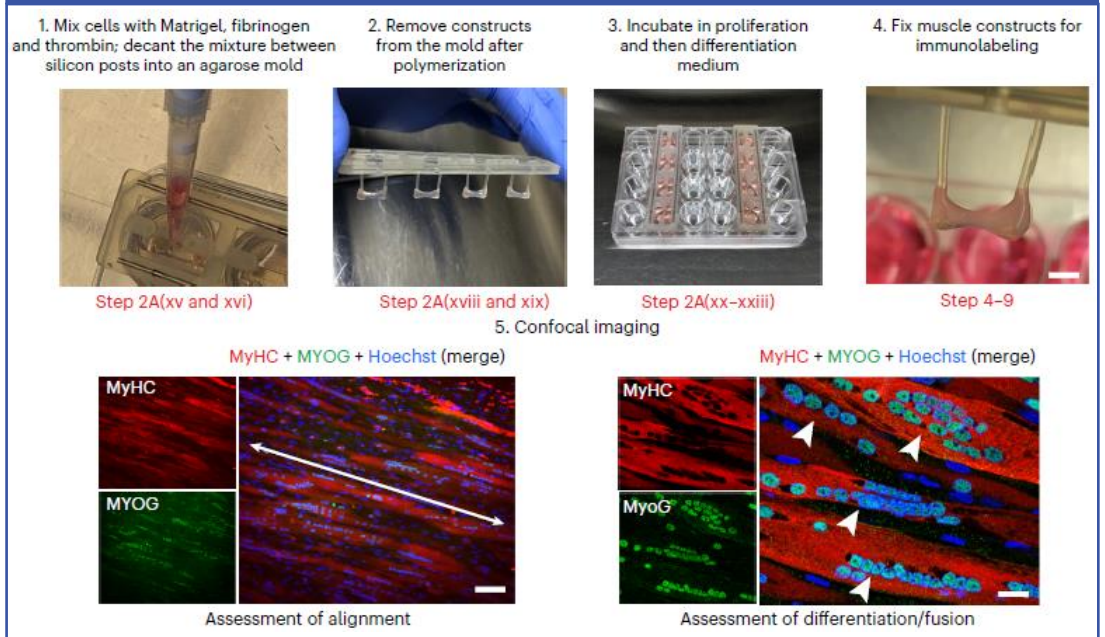
## Advantages of 3D culture systems

- Scaffolds/molds promote linear myotube formation with decreased variability in myotube morphology between experiments
- Extended culture longevity provides a platform to perform longitudinal studies
- Pharmacokinetic/pharmacodynamic studies are possible in a human cell model due to extended time in culture.
- Improved physiological relevance with better structural maturation; functional/force measurements possible

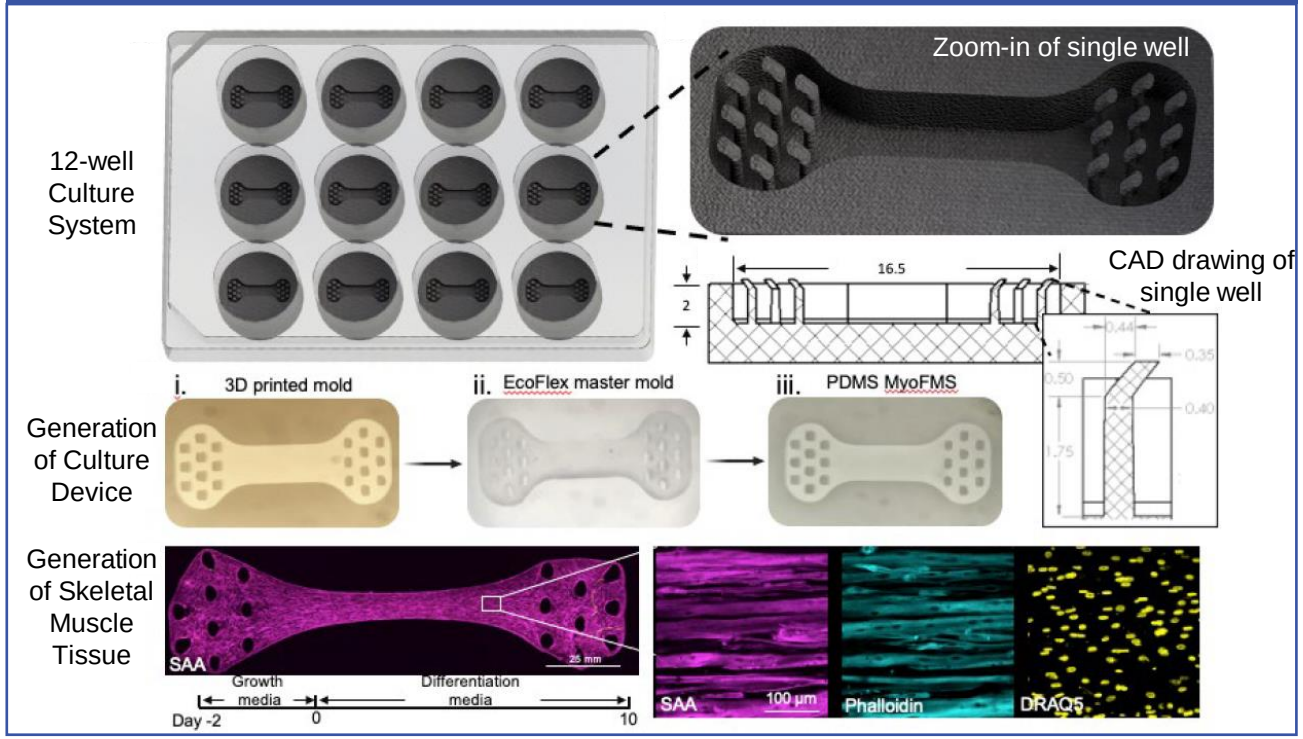
Smith, A.S. et al. *J Tissue Eng*. 2022

# 3D ENGINEERED MUSCLE TISSUES MIMIC IN VIVO PROPERTIES AND RESPOND TO STIMULATION

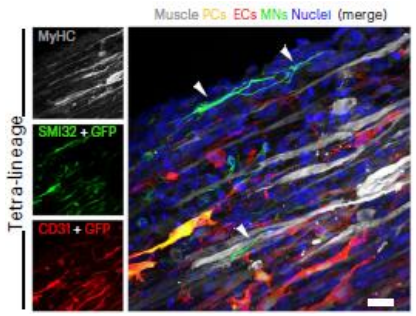
## Testing of Biomaterials and Scaffold with Human Myoblasts[1]



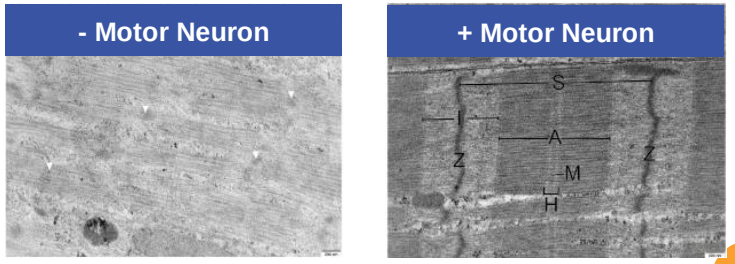

## 3D Model of MyoFMS Culture System[2]




Multi-lineage cultures which more closely mimic in vivo muscle composition are possible




Co-culturing 3D engineered tissue with motor neurons improve sarcomeric structure/maturity

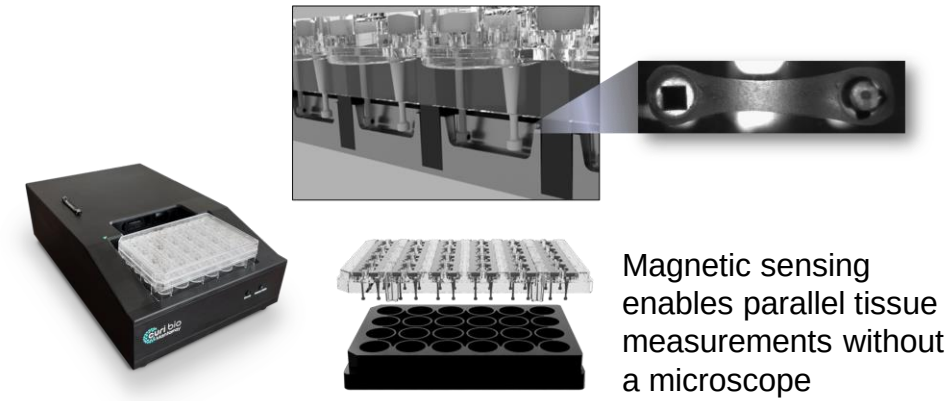



1. L. Pinton et al. *Nature Protocols*. 2023. 2. Nguyen C.T. et al. bioRxiv (preprint) doi.org/10.1101/2023.03.03.530083 (2023). (Left) Protocol for testing and quality control of tissue-derived myoblasts. **MyHC**, myosin heavy chain; **MYOG**, myogenin. (Right) MyoFMS device skeletal muscle tissue. **SAA**, sarcomeric α-actinin; **S**, sarcomere; **A**, A-bands; **M**, M-lines; **H**, H-zones.

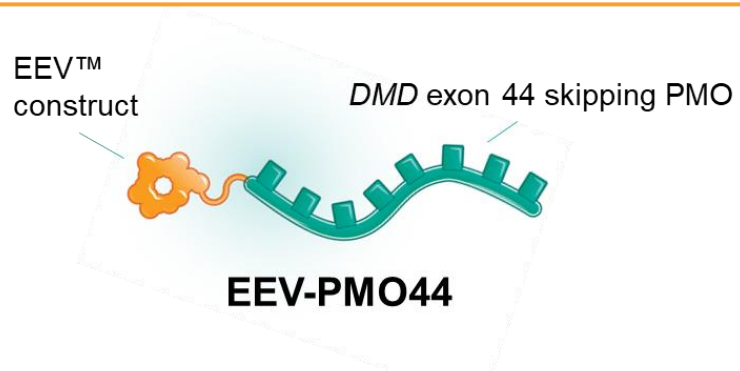
# DEVELOPMENT OF 3D EMT CULTURES

**OBJECTIVE:** To assess the utility of EMTs as an in vitro model system for examining the efficacy of a DMD exon skipping Endosomal Escape Vehicle (EEV)–PMO construct

- Culturing muscle cells is challenging because mature myotubes typically detach from their substrate within 1 week of initiating the differentiation regimen when cultured in a 2-dimensional (2D) matrix
  - A 3-dimensional (3D)–engineered skeletal muscle organoid system (i.e., engineered muscle tissue [EMT]) was developed to circumvent these challenges.[1]
- The Mantarray™ 3D EMT Contractility System has been shown to accurately depict defects in skeletal muscle cells derived from patients with DMD.



Magnetic sensing enables parallel tissue measurements without a microscope




- EEV-PMO44 is a *DMD* exon 44 skipping PMO conjugated to an EEV construct.
- Immortalized patient-derived skeletal myoblasts harboring an exon 45 deletion were engineered into EMTs (Δ45 DMD EMTs) with the Mantarray EMT Contractility System (Curi Bio, Seattle, WA).
- Δ45 DMD EMTs were exposed to varying doses of EEV-PMO44 (low, medium, high) for 24 hours, followed by compound washout.
- Δ45 DMD EMTs were assessed for DMD exon 44 skipping and dystrophin protein restoration 1, 4, and 6 weeks following compound removal.

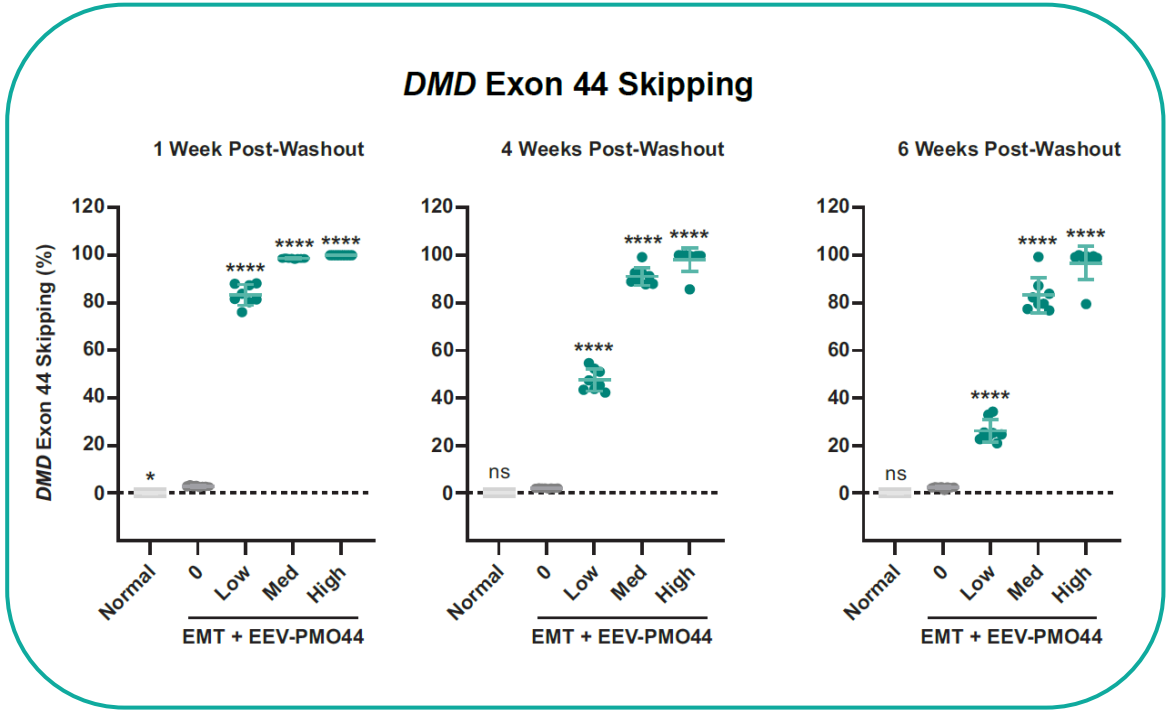


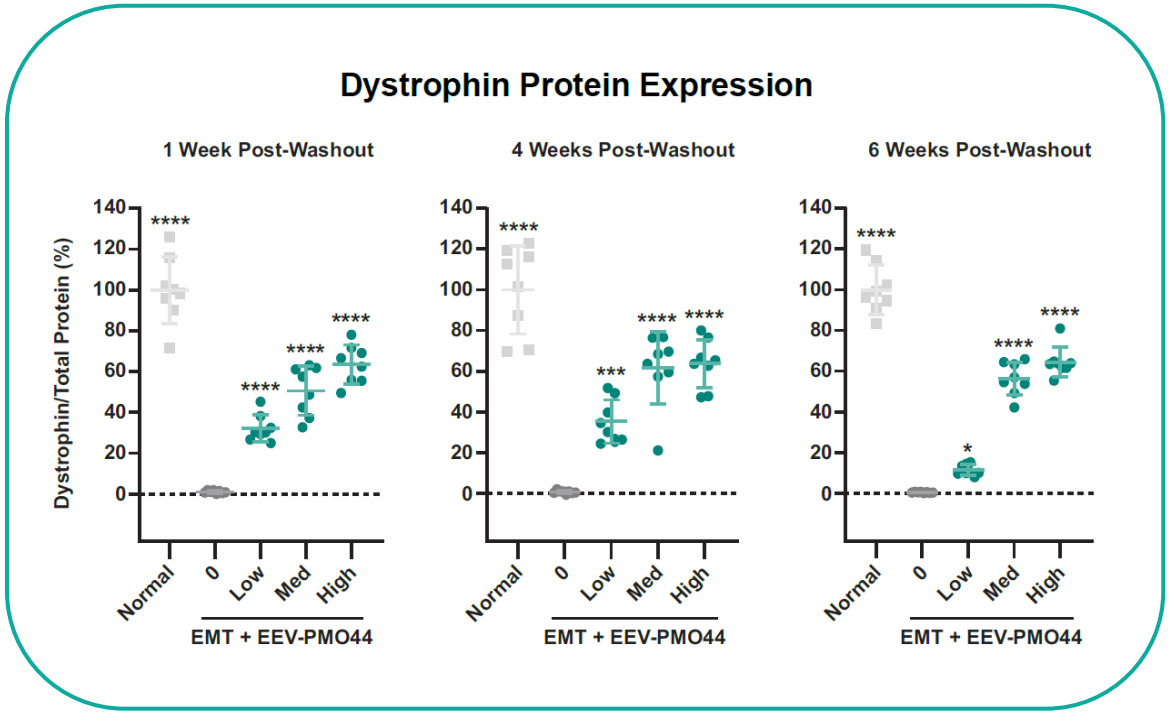


1. Smith AS, et al. J Tissue Eng. 2022. The Mantarray™ System was developed by Curi Bio (Seattle, WA). **DMD**, Duchenne muscular dystrophy; **EMT**, engineered muscle tissue; **PMO**, phosphorodiamidate morpholino oligomer.

# *DMD* EXON 44 SKIPPING AND DYSTROPHIN PROTEIN EXPRESSION

## Dose-dependent *DMD* exon 44 skipping and dystrophin protein expression were observed in 3D Δ45 DMD EMT cultures for up to 6 weeks post-washout following treatment with EEV-PMO44




The 3D organoid system allows for measuring EEV-PMO44 efficacy and duration of effect, demonstrating an advantage over traditional 2D culture systems.



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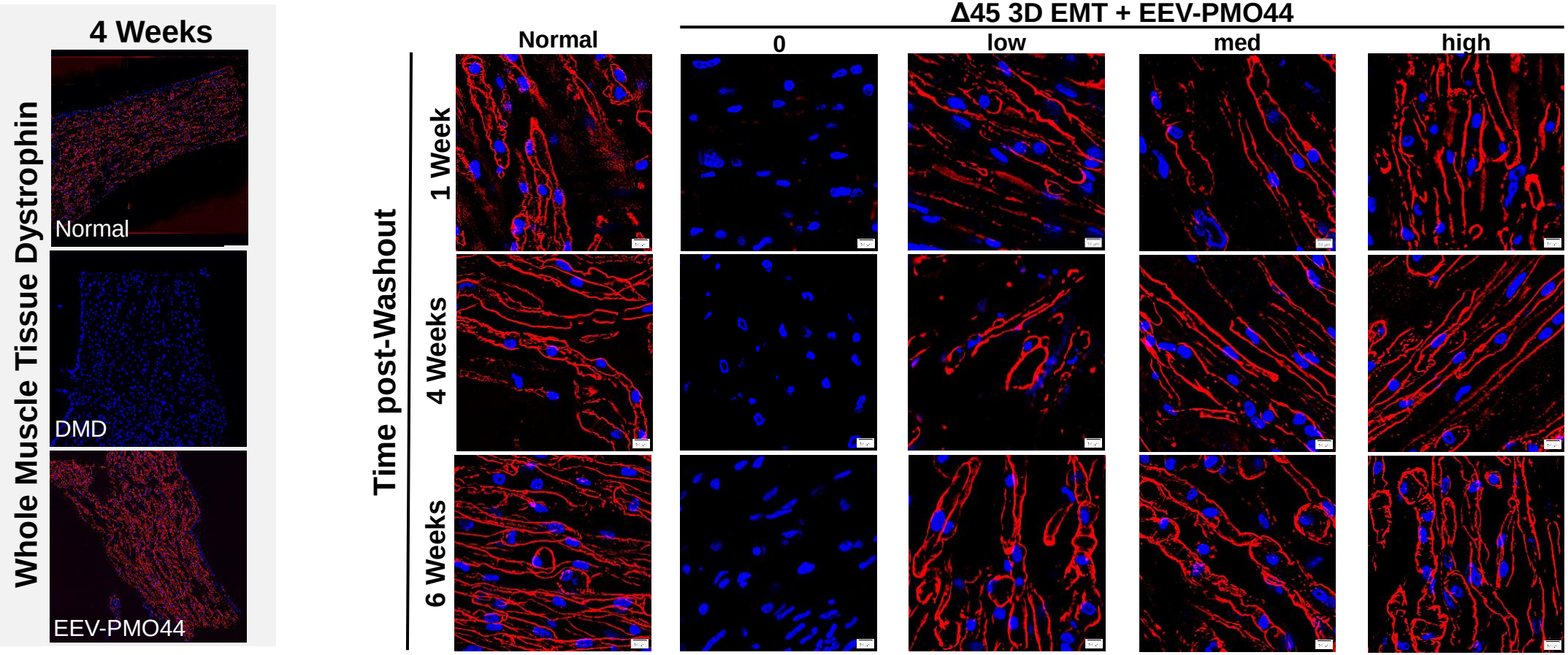
EMTs (N=8) were treated with EEV-PMO44 for 24 hours and analyzed at 1-, 4-, and 6-weeks post-washout. Normal cells from individuals without DMD were used as controls. Data are shown as mean ± SD; Ordinary 1-way ANOVA and Dunnett's multiple comparison test; *p<0.05, ***p<0.001, ****p<0.0001, in relation to untreated EMTs. **ANOVA**, analysis of variance; **DMD**, Duchenne muscular dystrophy; **EEV**, endosomal escape vehicle construct; **EMT**, engineered muscle tissue; **Med**, medium; **ns**, not significant; **PMO**, phosphorodiamidate morpholino oligomer.

# DYSTROPHIN RESTORATION (LONGITUDINAL)

## Longitudinal sections of EMT also showed broad dystrophin expression across the entire tissue sample following EEV-PMO44 treatment and proper localization to the sarcolemma



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EMTs (N=4) were treated with EEV-PMO44 for 24 hours and analyzed at 1-, 4-, and 6-weeks post-washout. Normal cells from individuals without DMD were used as controls. Red, dystrophin; blue, DAPI. (Left) Longitudinal images (40X stitched) of whole tissue treated with high dose EEV-PMO44 were examined 4 weeks post-washout. (Right) Longitudinal images (40X cropped) of EMTs. **DMD**, Duchenne muscular dystrophy; **EEV**, endosomal escape vehicle construct; **EMT**, engineered muscle tissue; **Med**, medium; **PMO**, phosphorodiamidate morpholino oligomer.

# CONCLUSIONS AND FUTURE DIRECTIONS

This 3D organoid system is a suitable model to demonstrate EEV-PMO construct efficacy and long-term duration of effect in vitro

- Consistent with previous studies in 2D cultures and animal models, these findings demonstrate the ability of the EEV™ platform to efficiently deliver exon skipping oligonucleotides to 3D-engineered skeletal muscle tissues as evident by exon skipping and dystrophin protein production.
- Dystrophin protein was observed in EMTs treated with EEV-PMO44 as early as 7 days post-washout, detectable for at least 6-weeks post-washout, and localized correctly to the sarcolemma.
- These results underscore the potential utility of this 3D culture system for the drug development process in Duchenne muscular dystrophy.

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