Physiological Function Assays for Muscular Dystrophy Therapeutics Screening with Stem Cell-derived Skeletal Myotubes

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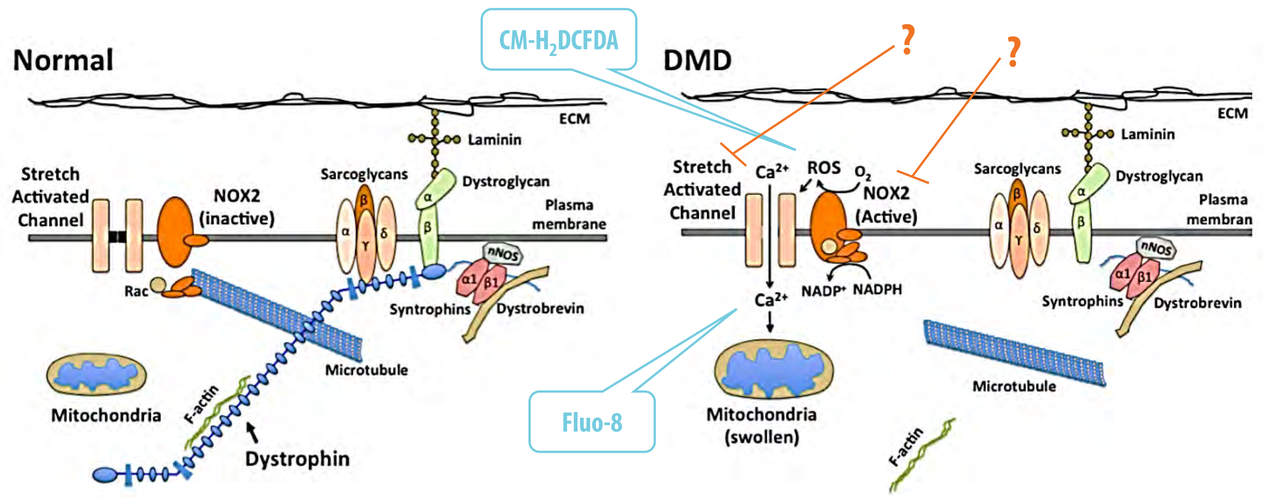
Introduction

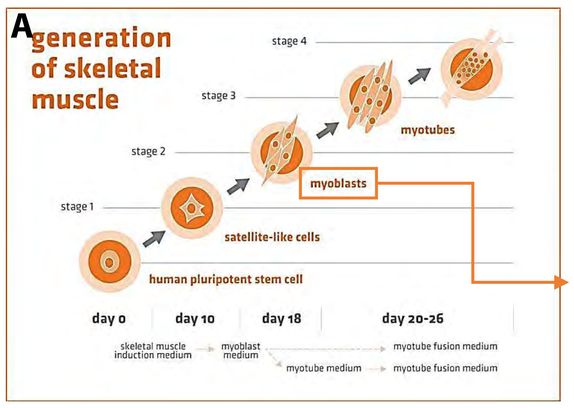
- Duchenne Muscular Dystrophy (DMD) is a X-chromosomal inherited disorder affecting 1 in 5000 males caused by mutations in the *DMD* gene which encodes a structural protein called dystrophin
- DMD is characterized by severe hypotonia, progressive muscle weakness and degeneration, and cardiovascular/respiratory impairments; **it is invariably fatal**
- Current DMD therapeutic screening methods are low-throughput, resource- and time-intensive, and may be invasive in nature
- Current screening methods have hampered drug discovery efforts and **there remains no cure for DMD**
- ***To address these issues, we developed and characterized stem cell-derived myotube-based models of DMD from patient samples, and are developing methods to test physiological function of hPSC-SkM using Kinetic Image Cytometry (KIC®)***

(Patho)physiology of DMD: the X-ROS Mechanism



- Loss of dystrophin in DMD increases stretch-induced activation of NOX2 to produce ROS, causing calcium influx via stretch-activated ion channels and, ultimately, mitochondrial dysfunction
- Using fluorescent indicators, we can measure ROS production (CM-H_2DCFDA) and calcium flux (Fluo-8)
- This mechanism also points towards potential druggable targets for muscular dystrophy (e.g. NOX2, stretch-activated calcium channels)

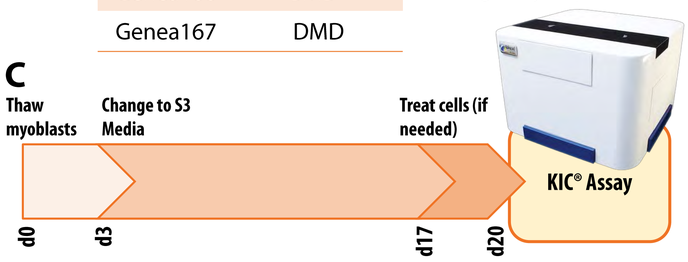
Materials, Methods, and Workflow



B

Cell Line	Disease Status
Genea002	Unaffected
Genea015	Unaffected
Genea166	BMD*
Genea167	DMD

*BMD (Becker's Muscular Dystrophy) is considered a milder disease phenotype caused by internal deletion of exons in the gene coding for dystrophin



Panel A: hPSC lines from different patients can be differentiated into skeletal muscle cells. After derivation of myoblasts, cells are cryopreserved and can be thawed with good viability, allowing for long-term banking of cells capable of being further matured into myotubes. ***C:*** Timeline for KIC® studies. Myoblasts are thawed into 96-well plates and allowed to recover for 3 days before the addition of Stage 3 (S3) Myotube Medium. Myotube formation proceeds over the next 2 weeks, at which point treatments can be added prior to KIC® assays for calcium and ROS production. ***B:*** hPSC-SkM cell lines from Genea Biocells used in the study. Genea166 and Genea167 were derived from iPSCs from the Coriell Institute specifically for this study. ***D*** (below)***:*** KIC® assay information.

D

Dye Loading → **KIC® Imaging with Contractile Stress Application** → **Image Analysis**

Ca^{2+} **Fluo-8**
(AAT Bioquest #21080)

- **Imaging:** 10 fps for 60 sec
- **Stress:** 50 msec-wide electrical pulses at 0.2 Hz (1 pulse per 5 sec)

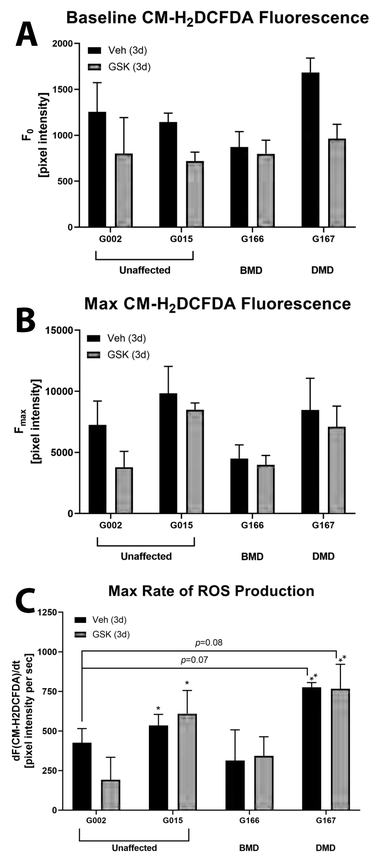
ROS **CM-H_2DCFDA**
(Thermo Fisher #C6827)

- **Imaging:** 10 fps for 30 sec
- **Stress:** 5 msec-wide electrical pulses at 40 Hz for 5 sec (tetanus)

Current: Manual analysis using ImageJ (**well-by-well measurements**)

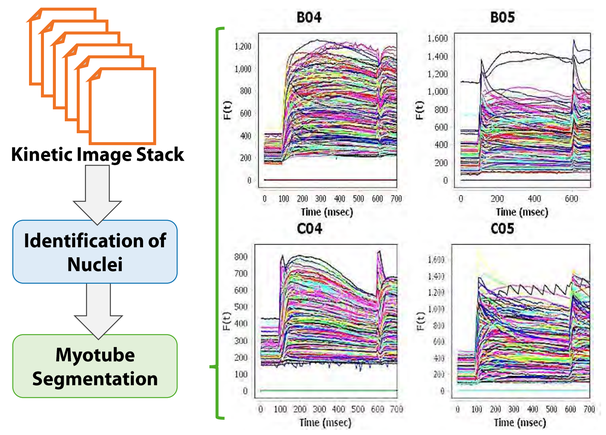
Ideal: Automated analysis using a plug-in algorithm *(in development)* for Vala's CyteSeer software (**cell-by-cell measurements**)

ROS Production



Baseline and maximum CM-H_2DCFDA fluorescence are reduced upon treatment with GSK275039, a NOX2 antagonist. ***A:*** 1 µM GSK treatment for 3d causes a decrease in baseline CM-H_2DCFDA fluorescence within each hPSC-SkM line compared to 0.1% DMSO vehicle (Veh). ***B:*** Likewise, GSK treatment causes a decrease in maximum CM-H_2DCFDA fluorescence compared to Veh. ***C:*** **The rate of ROS production during electrical stimulation is elevated in DMD hPSC-SkM compared to unaffected SkM, but GSK treatment has variable effects within cell lines.** The maximum ROS production rate was determined by finding the maximum slope over the course of 1 sec during the 5 sec tetanic stimulation procedure. GSK treatment seems to modulate the basal and maximum ROS levels, but not necessarily the rate at which ROS is generated.

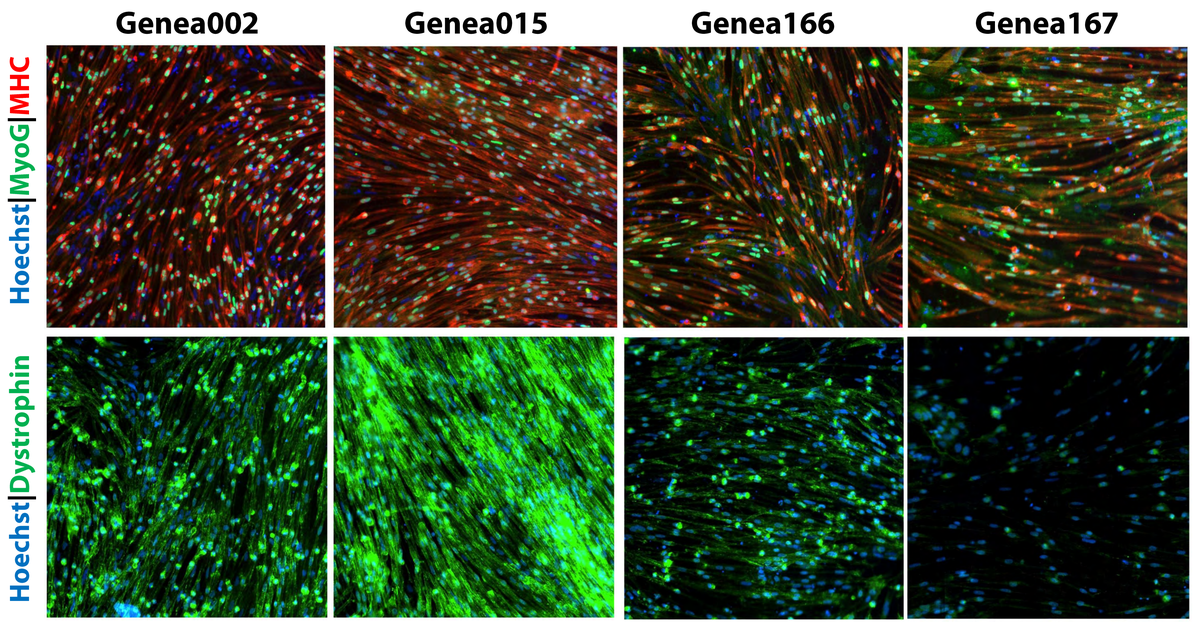
Calcium Flux



Single-cell calcium measurement work-flow with CyteSeer®. The traces to the left are examples of single calcium transients measured for each "cell" in the field of view, elicited by electrical stimulation generated by CyteSeer® using the basic work-flow below. Parameters characterizing the kinetics of calcium transients (e.g. peak amplitude, upstroke/downstroke velocity, duration measurements) are calculated using the software. **These data are still being analyzed and the algorithm is still being refined to better segment myotubes.**

◀ *Bars/error lines represent mean±SD. Statistical analysis by 2-way ANOVA followed by Tukey's multiple comparison test; explicit p-values are reported for non-significant differences (approaching significance) between G002 Veh and indicated group; *p<0.05, **p<0.01, compared to G002 GSK.*

Cell Line Characterization



- Biomarkers for lineage commitment and structural maturity were evident in control lines by S3d3; however, KIC® assays were performed at later timepoints to allow for further myotube maturation.
- Note evidence of impaired myogenesis and deficiencies in dystrophin in dystrophic lines compared to unaffected controls.

Conclusions and Future Directions

- We have successfully generated structurally-mature myotubes from dystrophic donors from iPSCs for use in these functional assays
- This model of DMD recapitulates several disease-specific defects, including impaired myogenesis (confirmed by immunofluorescence, impaired mitochondrial function (by ROS output), and aberrant calcium signaling
- The use of iPSC-derived myotubes obviates the time and effort needed to breed animals and maintain colonies for compound testing, and provides a bankable source of human cells amenable to assay scale-up for high-throughput screens
- High-throughput physiological screens of muscle function will require the ability to process complex kinetic image stacks in an automated fashion (e.g. with Vala's CyteSeer®)
- The variability in responses between unaffected cell lines may be attributed to the genetic background of each donor from whom the stem cells were derived
- Genomic editing of the stem cell lines through CRISPR/Cas9 (either by correcting *DMD* mutations in dystrophic iPSC lines or by mutating *DMD* in unaffected iPSC lines) would theoretically control for this genetic variability and represents an attractive future direction
- Vala Sciences, Inc. has successfully created analytical algorithms to measure contractility in cardiomyocytes; adaptation of these algorithms to myotubes is underway to further increase the amount of functional data

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