GENERATION OF HUMAN IPSC-DERIVED DUCHENNE MUSCULAR DYSTROPHY SKELETAL MYOCYTES SUITABLE FOR 3D FUNCTIONAL STUDIES AND INVESTIGATING METHODS FOR DYSTROPHIN RESTORATION

Authors

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Abstract

Skeletal myocytes play a number of roles in many biological processes ranging from limb movement to the regulation of nutritional homeostasis and are implicated in the pathophysiology of a variety of diseases such as muscular dystrophies and metabolic disorders. There is a pressing need for reliable models of mature human skeletal muscle to permit investigations into physiological and disease mechanisms, and to facilitate the generation of new therapeutics.

We have developed a deterministic cell programming technology, optimised inducible overexpression (opti-ox*) that enables tightly controlled expression of transcription factors (TF), improving cellular reprogramming approaches for the differentiation of human induced pluripotent stem cells (hiPSCs). Through opti-ox mediated TF overexpression, we have generated ioSkeletal Myocytes* that homogeneously express the key proteins of the myofilaments, desmin, dystrophin and titin, and form striated and multinucleated myocytes that are functionally active.

Furthermore, ioSkeletal Myocytes can form functional 3D skeletal muscle microtissues using the MUSbit™ microchip, where twitch and tetanus responses can be observed and become stronger over time, and contraction in response to electrical stimulation can be inhibited or stimulated by the addition of compounds.

We employed CRISPR/Cas9 gene editing to introduce a deletion of exon 44 (Del Ex44) or exon 52 (Del Ex52) in the DMD gene of wild type ioSkeletal Myocytes to recapitulate the genetics of Duchenne muscular dystrophy (DMD). The generation of DMD deletions in the ioSkeletal Myocytes results in inhibited dystrophin expression at both the transcript and protein level but does not impair their ability to form myocytes.

Restoration of dystrophin mRNA and protein expression has been demonstrated using ASO-mediated exon skipping. Utilising the MUSbit microchip, we have observed impaired functional activity of DMD ioSkeletal Myocytes including reduced twitch and tetanus contraction in response to electrical stimulation.

Overall, we have demonstrated the ability to generate a reproducible isogenic system for the investigation of Duchenne muscular dystrophy. This novel system has the potential to reveal new in vitro functional phenotypes to improve drug screening specificity and accelerate drug development.

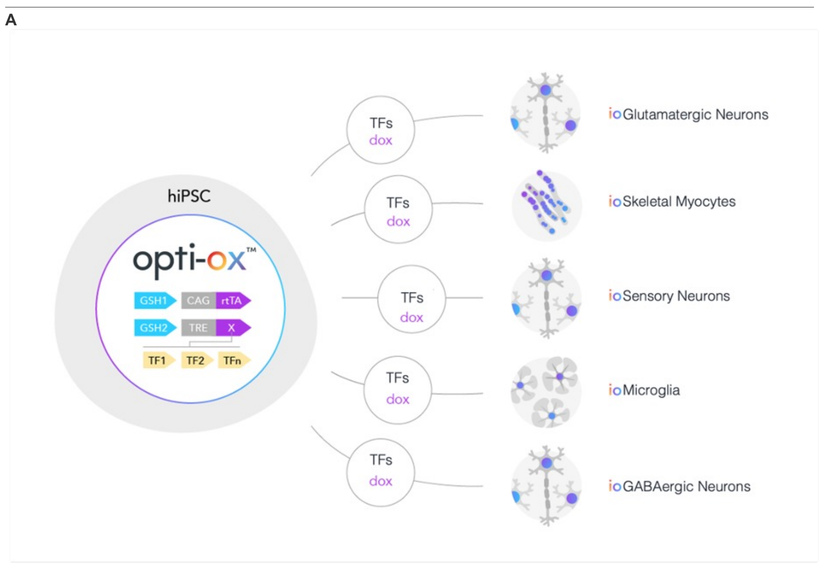
1. Precise programming of iPSCs into defined human cell types

opti-ox technology for the deterministic cell programming of human iPSCs into defined human cell types.

opti-ox is a dual cassette Tet-ON system that ensures tightly controlled and homogeneous expression of programming TFs by preventing silencing of the inducible expression cassette after genetic engineering of hiPSCs.

TF expression through opti-ox has been demonstrated to generate cell types from all three germ layers in a robust, scalable manner.

Additionally, we have developed a Discovery Platform that allows for the identification of core TF networks that drive cell fate acquisition from pluripotent stem cells.



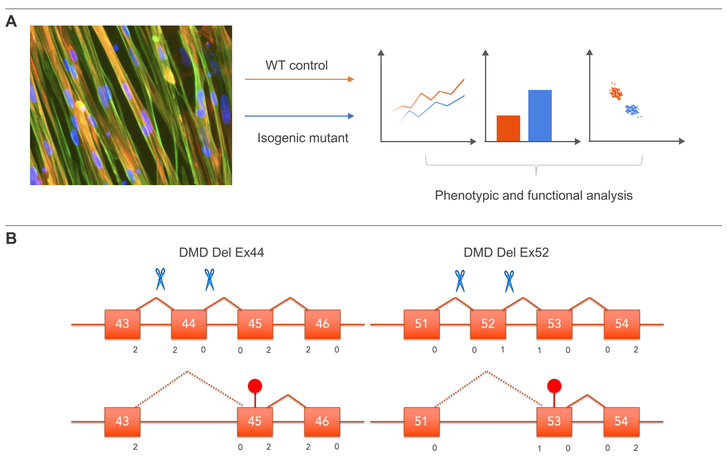
2. Development of an isogenic model of Duchenne muscular dystrophy

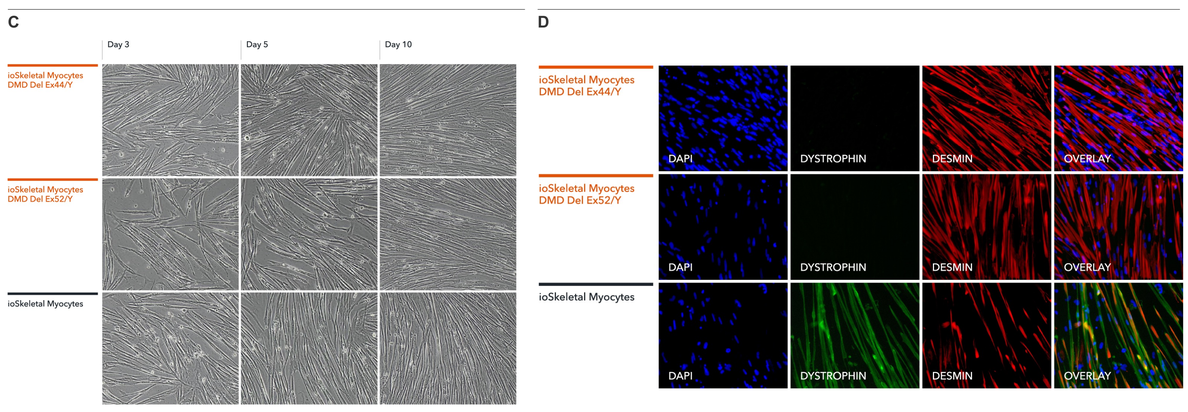
ioSkeletal Myocytes with DMD-relevant exon deletions.

(A) We have developed ioSkeletal Myocytes, hiPSC-derived skeletal myocytes, using opti-ox technology. The wild type (WT) parental iPSC line was engineered to introduce exon deletions in the dystrophin gene, enabling phenotypic and functional characterisation of the derived disease models compared to their isogenic WT control.

(B) CRISPR/Cas9 mediated gene engineering was used to delete either exon 44 (DMD Del Ex44) or 52 (DMD Del Ex52).

(C) DMD ioSkeletal Myocytes demonstrate similar morphology to the isogenic control but have impaired dystrophin expression (D) when assessed by immunocytochemistry (day 10 of programming).



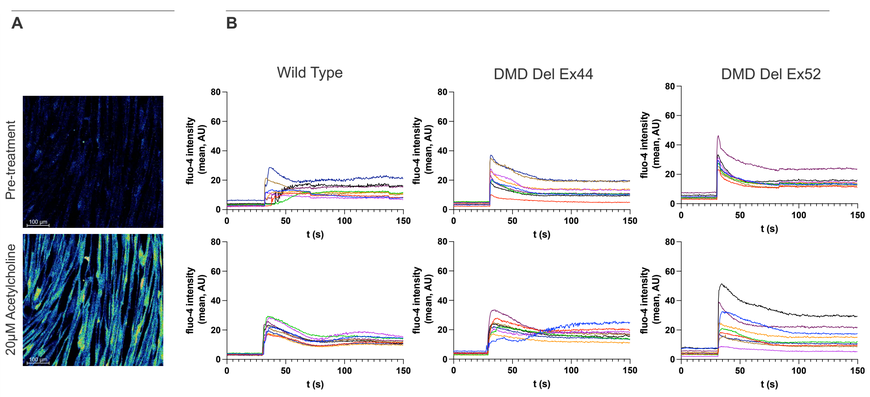


3. Skeletal myocyte contraction assessment in monolayer culture utilising calcium binding dyes

Skeletal myocytes demonstrate functional responses in monolayer culture.

(A) On day 10 post-revival, ioSkeletal Myocytes WT and DMD disease models were loaded with Fluo4-AM (pre-treatment) for the assessment of calcium transients during contractile responses following stimulation with acetylcholine (20µM).

(B) WT and DMD show functional responses to acetylcholine. Each graph represents an individual well; coloured lines represent different regions of interest.



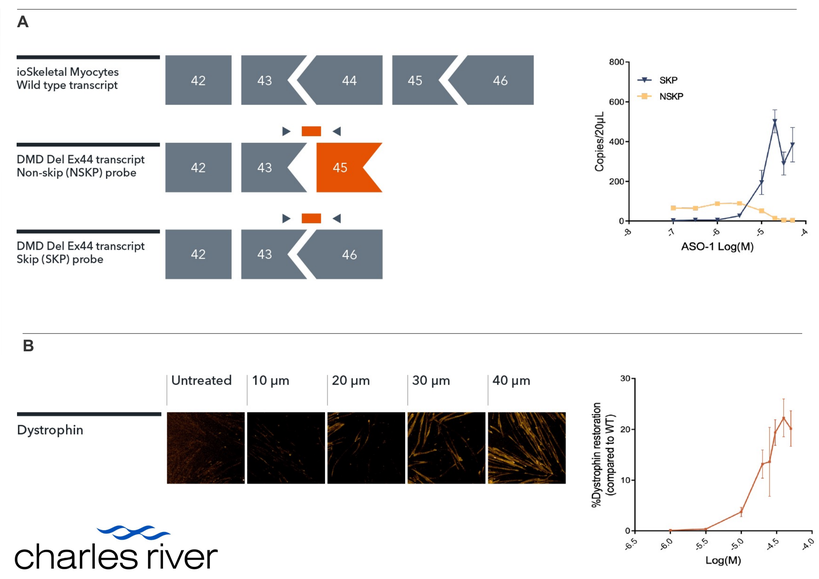
4. ASO mediated restoration of dystrophin expression in ioSkeletal Myocytes DMD Exon 44 Deletion

ioSkeletal Myocytes DMD disease model cells can be used as a platform for antisense oligonucleotide (ASO) assessment.

ioSkeletal Myocytes DMD Del Ex44 were treated with an exon 45 skipping ASO at day 4 post-revival.

(A) A ddPCR assay was designed to amplify the region coding exons 43-46 (successful exon skipping, SKP) or exons 43-45 (non-skip, NSKP). At day 7 a concentration-dependent increase in the amount of SKP transcript and a decrease in the NSKP transcript were observed, indicating that ASO treatment was successful in creating an in-frame mRNA transcript for dystrophin.

(B) Dystrophin protein expression was assessed by immunocytochemistry at day 7 and was seen to increase in an ASO concentration-dependent manner.



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5. Development of 3D skeletal muscle microtissues that mature over 14 days

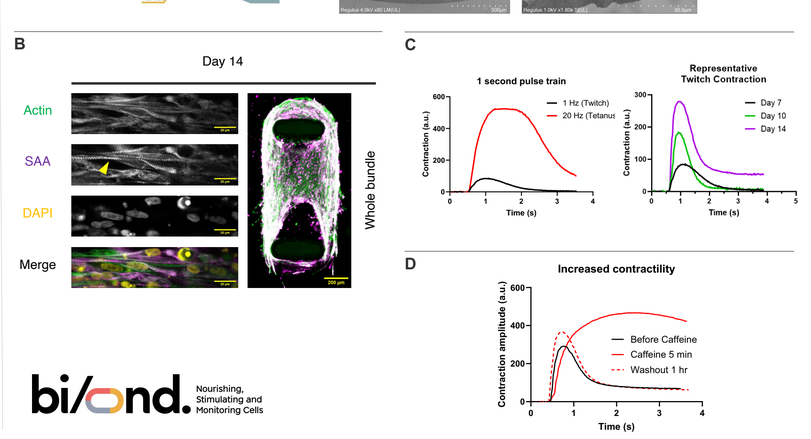
Muscle bundles become stronger and more mature over time.

(A) ioSkeletal Mycoytes were successfully cultured in 3D on Bi/ond's MUSbit microchip, which includes pillars for anchoring muscle cell bundles. Scanning electron microscopy demonstrates successful microtissue formation and muscle fiber formation (green arrow).

(B) Immunocytochemistry after 14 days of culture demonstrates expression of key muscle markers and clear sarcomere formation (yellow arrow).

(C) Twitch and tetanus forces can be observed in 3D muscle bundles. Contractile forces increase over time indicating an increase in muscle maturity and strength.

(D) Contractility increases when the bundle is electrically stimulated following addition of caffeine, which stimulates Ca^{2+} release from the sarcoplasmic reticulum.



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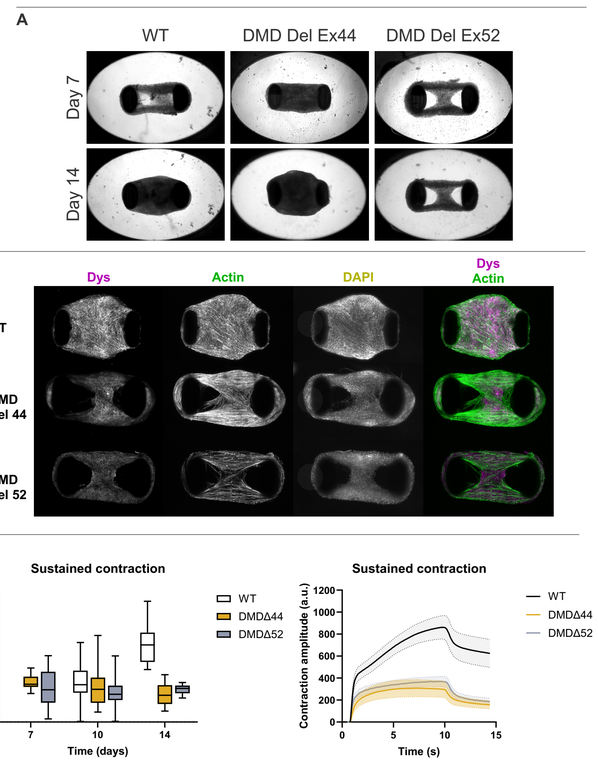
6. DMD ioSkeletal Myocytes demonstrate reduced contractile function in 3D microtissues

Functional characterisation of DMD ioSkeletal Myocytes in 3D.

(A) ioSkeletal Myocytes DMD Exon Deletion disease model cells can form 3D microtissues on the MUSbit microchip.

(B) 3D microtissues show robust muscle microtissue formation, with reduced dystrophin expression observed in DMD cells.

(C) DMD 3D microtissues demonstrate reduced twitch, tetanus, and sustained contraction as cells mature over time.



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Summary & conclusions

We have developed human iPSC-derived ioSkeletal Myocytes, which can be used as a tool to develop muscle related disease models. We have used CRISPR/Cas9 gene engineering to generate Duchenne Muscular Dystrophy models with disease-relevant exon deletions.

DMD ioSkeletal Myocytes are able to programme to a skeletal muscle identity with a muscle morphology and expression of key muscle markers. Importantly, DMD ioSkeletal Myocytes have reduced expression of dystrophin at the protein level.

We also demonstrate that WT and DMD ioSkeletal Myocytes can be used for functional assessment in monolayer culture through calcium imaging and ASO-mediated dystrophin restoration. Furthermore, we demonstrate that WT and DMD ioSkeletal Myocytes can be used to develop 3D muscle bundles that reveal key DMD phenotypes.