Discovery of Novel Small-Molecule Treatment Options for FSHD

Geese Marcus[1], Ermann Monika[1], Schneider Martin[1], Monecke Sebastian[1], Mosblech Alina[1], Dickie Tony[1], Bayerlova Michaela[1], Kaever Alexander[1], Loke Pui[1], James Timothy[1], De Maeyer Joris[2]

[1]Evotec SE, Hamburg, Germany;
[2]Facio Therapies BV, Leiden, The Netherlands

Introduction

FSHD is caused by inefficient epigenetic repression of the *DUX4* gene. *DUX4* encodes a transcription factor whose expression is normally restricted to early embryonic development. Gain-of-function of DUX4 in muscle tissue of FSHD patients initiates a transcription cascade ultimately resulting in overt muscle pathology. Repressing the expression of DUX4 offers a valid approach for therapeutic intervention. Given the complex regulatory mechanisms involved in DUX4 repression, the use of primary patient-derived myotubes is warranted for optimal translatability.

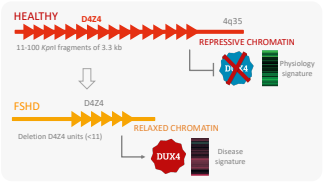

Figure 1 - DUX4 de-repression is a validated marker of epigenetic changes in FSHD muscle

Sensitive DUX4 ICC detection method

- *In vitro*, DUX4 shows a variegated expression pattern in only a small fraction of myonuclei
- Drug discovery efforts have been hampered by the absence of robust methods for detection of endogenous DUX4
- We developed a screening-compatible method enabling rapid and sensitive microscopic detection of sporadic DUX4 expression in primary patient-derived myocytes

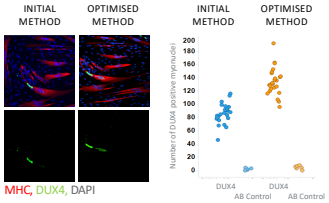

Figure 2 –A method to robustly detect rare DUX4-positive nuclei in myotube cultures from FSHD patients was developed. Staining is restricted to few nuclei of myotubes from FSHD patients while no staining is observed in myoblasts or in myotubes from healthy donors. The DUX4-positive nuclei often appear in clusters showing a gradient of staining intensity within a cluster as described in the literature. More DUX4- positive nuclei are detected using the optimized method.

High-content screening in primary cells

- We developed an HCS assay in 384-well format based on script-based image analysis
- The Assay uniquely enables quantifying endogenous DUX4 expression together with myotube fusion and cell toxicity in primary patient-derived muscle cells
- A diverse library of 90,000 novel small molecules was screened in the automated HCS assay

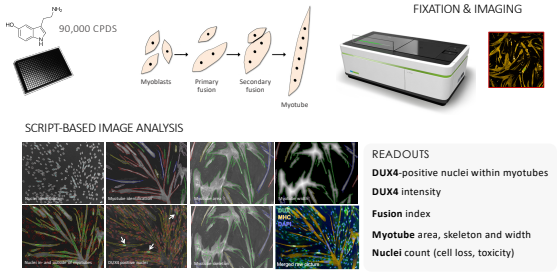

Figure 3 – Schematic representation of the high-content screening workflow. Primary FSHD myoblasts are seeded in 384 well plates. 24h later, the medium is changed into differentiation medium and small-molecule compounds are added. After 3 days of differentiation, cells are fixed and imaged on an Opera Phenix™ imaging system. Quantitative readouts are obtained using a script-based analysis. Assay performance is very good with Z' values above 0,6 for DUX4 or 0,8 for fusion inhibition.

Mitigating false-positive risk

- *In vitro*, DUX4 expression is triggered during myotube fusion
- Fusion Index differences are directly reflected in DUX4 readout in the fusion-dependent assay
- Our HCS assay allows sensitive monitoring of fusion efficiency, which is imperative to avoid artefacts caused by inhibition of fusion, i.e. the natural trigger of DUX4 expression
- Previously reported DUX4 repressors were deprioritized because they affected myotube fusion

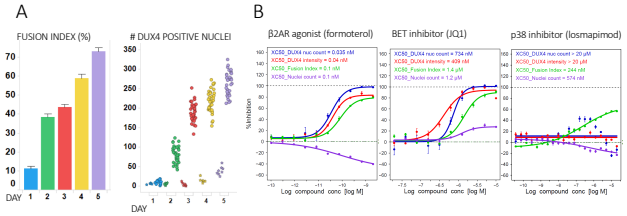

Figure 4 – (A) The HCS assay is fusion-dependent with DUX4 expression being triggered during myotube fusion. When cultured in differentiation medium, the fusion index (fraction of nuclei inside myotubes) increases as primary myotubes are formed. This process triggers DUX4 expression. (B) Concentration-response profiles obtained in the HCS assay with a number of mechanisms previously reported to inhibit DUX4 in FSHD myotubes. Each of these mechanisms concentration-dependently inhibited myotube fusion, implying a risk of false-positive DUX4 inhibition.

Identification of novel DUX4 repressors

- 606 confirmed DUX4 inhibitors identified
- Selected compound series are being progressed through H2L and LO

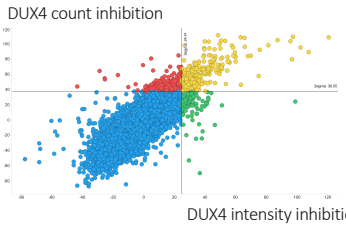

Figure 5 - Correlation of the primary readouts obtained in the screening campaign for fusion-neutral compounds. The X-axis shows the percentage inhibition of the intensity of the DUX4 signal while the Y-axis shows the percentage inhibition of the number of DUX4-positive myonuclei. The horizontal and vertical line represent the 3sigma criterion used for hit calling.

CK1 inhibitors block DUX4 expression and normalize toxic DUX4 transcriptome

- Casein Kinase 1 was validated as the target of one of the discovered compound series
- CK1 inhibitors strongly repress DUX4 expression without affecting fusion
- CK1 inhibitors normalize the FSHD transcriptome in primary myotubes

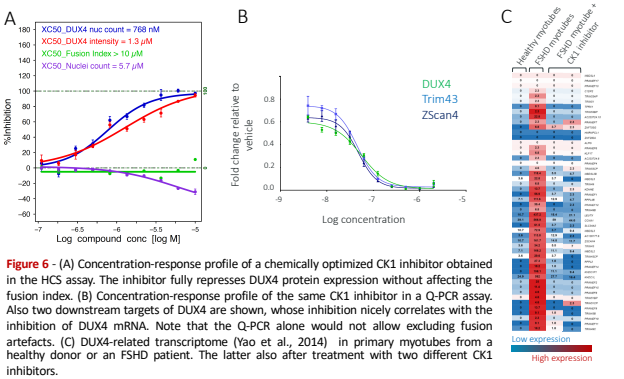

Figure 6 - (A) Concentration-response profile of a chemically optimized CK1 inhibitor obtained in the HCS assay. The inhibitor fully represses DUX4 protein expression without affecting the fusion index. (B) Concentration-response profile of the same CK1 inhibitor in a Q-PCR assay. Also two downstream targets of DUX4 are shown, whose inhibition nicely correlates with the inhibition of DUX4 mRNA. Note that the Q-PCR alone would not allow excluding fusion artefacts. (C) DUX4-related transcriptome (Yao et al., 2014) in primary myotubes from a healthy donor or an FSHD patient. The latter also after treatment with two different CK1 inhibitors.

CK1 inhibitors reduce DUX4 expression in a patient-derived xenograft model

- A xenograft mouse model was developed using patient-derived primary FSHD myoblasts
- A CK1 inhibitor achieving muscle exposure above the *in vitro* DUX4 EC_{50} value after oral dosing was selected for a Proof of Principle study
- Oral administration of the CK1 inhibitor dose-dependently inhibited expression of DUX4 and DUX4 downstream target genes without affecting myogenic markers

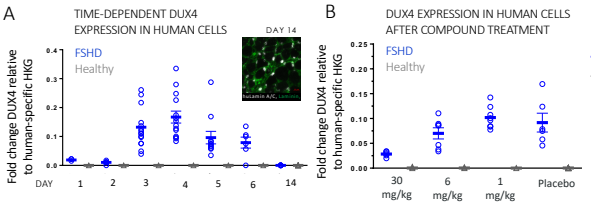

Figure 7 - A $BaCl_2$ solution of primary muscle cells, with retained *in vitro* differentiation capacity, was injected into the tibialis anterior of NOD SCID mice. Cells from a healthy or FSHD donor were injected in the contralateral site. (A) Mice were sacrificed after selected time points, xenografts dissected and analyzed microscopically and via Q-PCR. The human muscle cells engrafted in the murine tissue, as confirmed by staining with human lamin A/C (shown for day 14). DUX4 expression peaked at day 4. (B) Primary muscle cells were injected as described in A, after which mice were treated with oral doses of a CK1 inhibitor twice daily for 4 days (not shown), or during the last 24h. The CK1 inhibitor dose-dependently reduced the level of DUX4 mRNA in the human xenograft.

Conclusions

- We established a unique HCS platform enabling automated quantification of DUX4 expression while simultaneously monitoring fusion efficiency to weed out potential artefacts.
- Multiple novel chemical scaffolds repressing DUX4 without affecting fusion were identified.
- CK1 was identified as the target of one of the compound series.
- In a human xenograft based on primary FSHD cells, *in vivo* proof of principle was demonstrated.

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