Towards a muscle-targeted delivery of antisense agents against DUX4

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GOAL OF THE STUDY

In a therapeutic approach for FSHD, Antisense Oligonucleotides (ASOs) have been developed against the 3'UTR of DUX4 mRNAs. However, the use of ASOs is currently limited because of their restricted tissue delivery, lack of tissue selectivity and rapid clearance since systemic administration mostly results in their capture by the liver and kidneys.

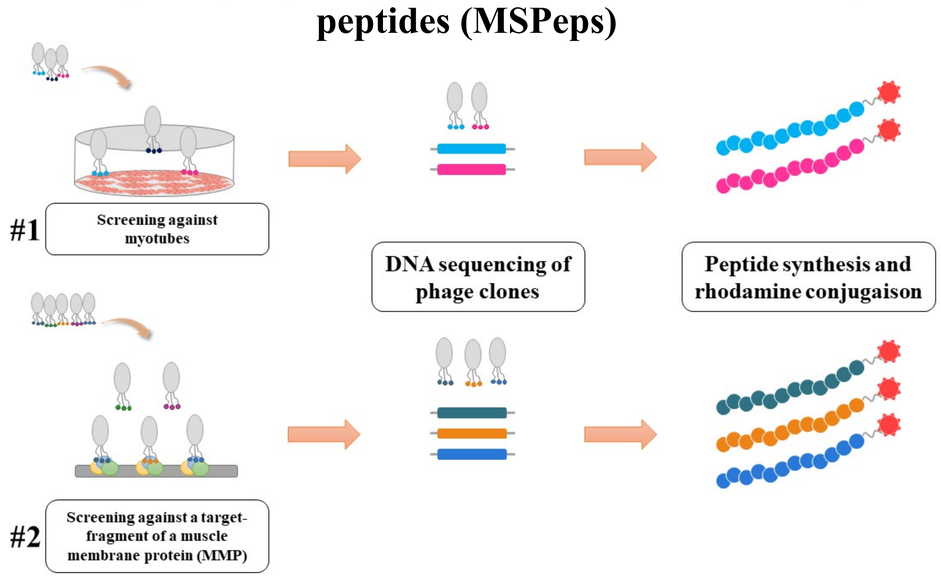
To improve ASO transport in the bloodstream and delivery into skeletal muscle, the first step of our project was **to define muscle-specific ligands specifically binding with high affinity to human skeletal muscle surface proteins and to their mouse homologues** (for later testing in mouse pre-clinical model).

To this aim, phage display peptide libraries were screened against (#1) human myotubes and (#2) a purified target-protein fragment synthesized based on studies *in silico* of a muscle membrane protein (MMP).

The 5 most promising phage clones were sequenced, and the encoded peptides (MSPeps) synthesized with Rhodamine conjugation for testing in cell culture *in vitro*.

METHOD

Screening of a phage display library to select muscle-specific peptides (MSPeps)



MSPeps selected against myotubes

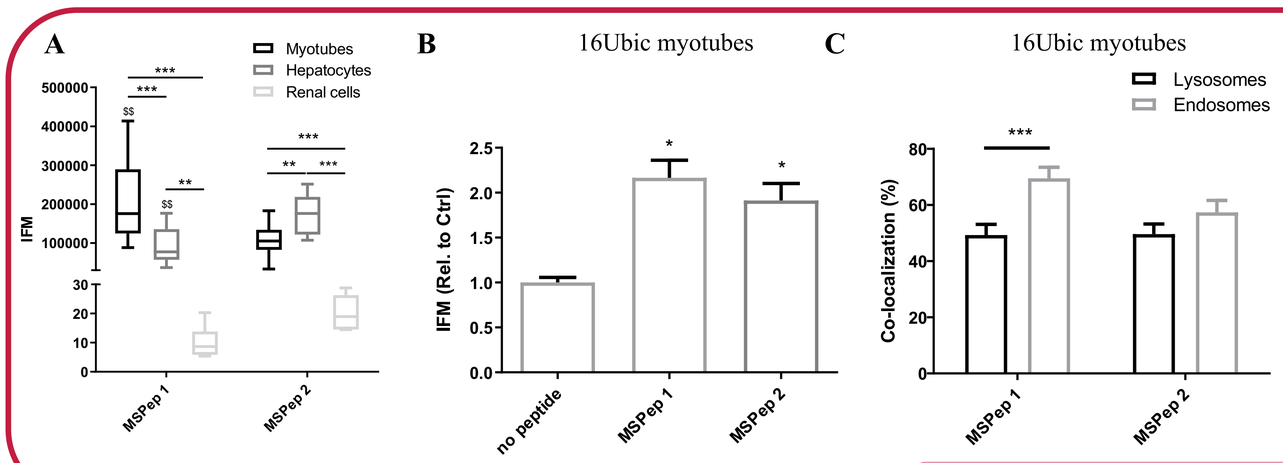


Figure 1 : Endocytosis and co-localization of MSPep 1 and 2 selected against myotubes. (A) Endocytosis analysis of MSPep 1 and 2 after 2hours of incubation with human myotubes (16Ubic), human hepatocytes (HepaRG) and human embryonic renal cells (HEK293). Results of the intensity of fluorescence measured (IFM) represented as BoxPlots, $$p<0.01 vs MSPep2 ; **p<0.01 et ***p<0.001 as indicated; Two-way ANOVA followed by Holm-Sidak test for multiple comparison, n=2, 10 microscopic fields analyzed per condition. **(B)** Lysotracker intensity of fluorescence (relative to control) following incubation with MSPep 1 and 2. Histograms represented as mean ± SEM, *p<0.05 vs no peptide; One-way ANOVA, n=2, 10 microscopic fields analyzed per condition. **(C)** Co-localization of MSPep 1 and 2 with lysosomes or early endosomes (measured with Lysotracker and BacMam early respectively). Histograms of co-localization represented as Manders coefficient ± SEM, ***p<0.001 as indicated; Two-way ANOVA, n=2, 10 microscopic fields analyzed per condition. Manders coefficient M1 and M2 were determined with JACoP plugin of ImageJ.

PRELIMINARY DATA

MSPeps selected against MMP

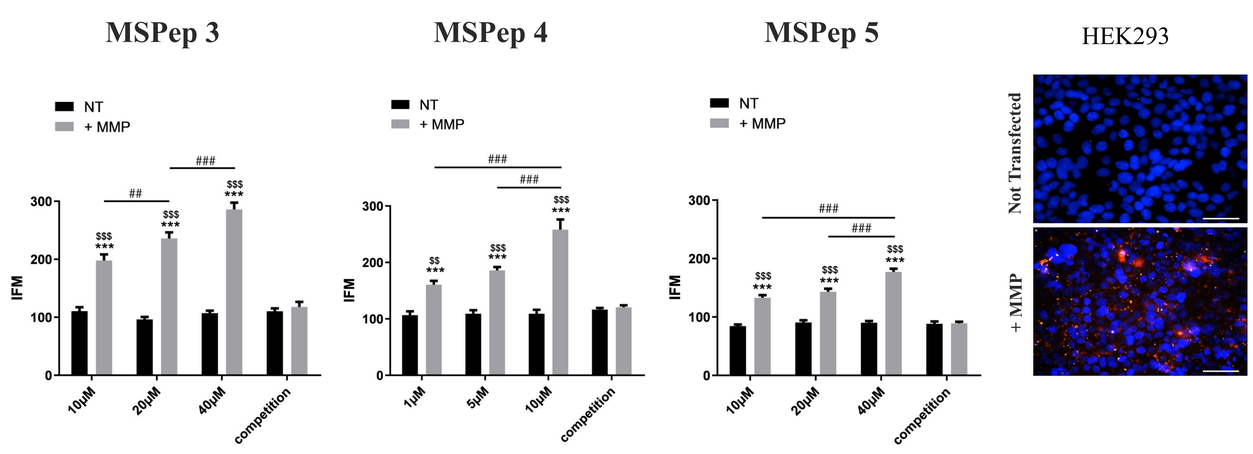


Figure 2: Evaluation of MSPep specificity on HEK293 cells transfected or not (NT) with a plasmid encoding MMP.
Left panel - HEK293 cells were transfected or not with a MMP expression vector (+MMP), and 24h later the indicated MSPeps were added for 2hours at 37°C in the cell culture medium. For binding competition, peptides (10µM) were preincubated with target-MMP (20µM). Histograms of the intensity of fluorescence measured (IFM) represented as mean ± SEM; ***p<0.001 vs NT, $$p<0.01 and $$$p<0.001 vs competition, ##p<0.01 and ###p<0.001 as indicated; Two-way ANOVA followed by Holm-Sidak test for multiple comparison, n=2, 10 microscopic fields analyzed per condition.
Right panel - Representative field of MSPep 3 40µM. Scale = 50µm .

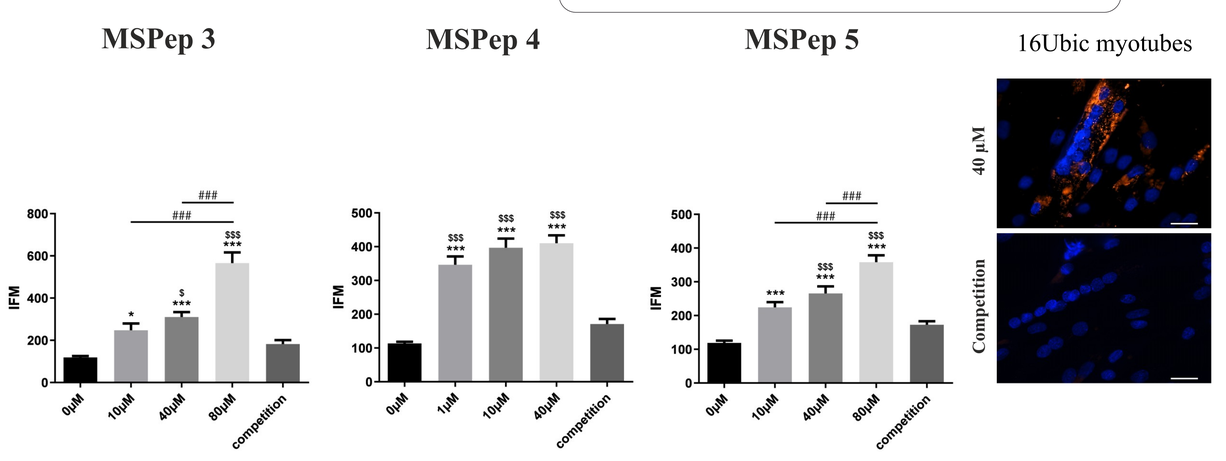


Figure 3: Evaluation of MSPep specificity on human myotubes.
Left panel - The indicated MSPeps were incubated 2h at 37°C in culture medium of human myotubes (16Ubic). For binding competition, peptides (20µM) were pre-incubated with target-MMP (40µM). Histograms of the intensity of fluorescence measured (IFM) represented as mean ± SEM; ***p<0.001 and *p<0.05 vs 0µM, $$$p<0.001 and $p<0.05 vs competition, ###p<0.001 as indicated; One-way ANOVA followed by Holm-Sidak test for multiple comparison, n=2, 10 microscopic fields analyzed per condition.
Right panel - Representative field of MSPep 3 40µM. Scale = 25µm .

CONCLUSION

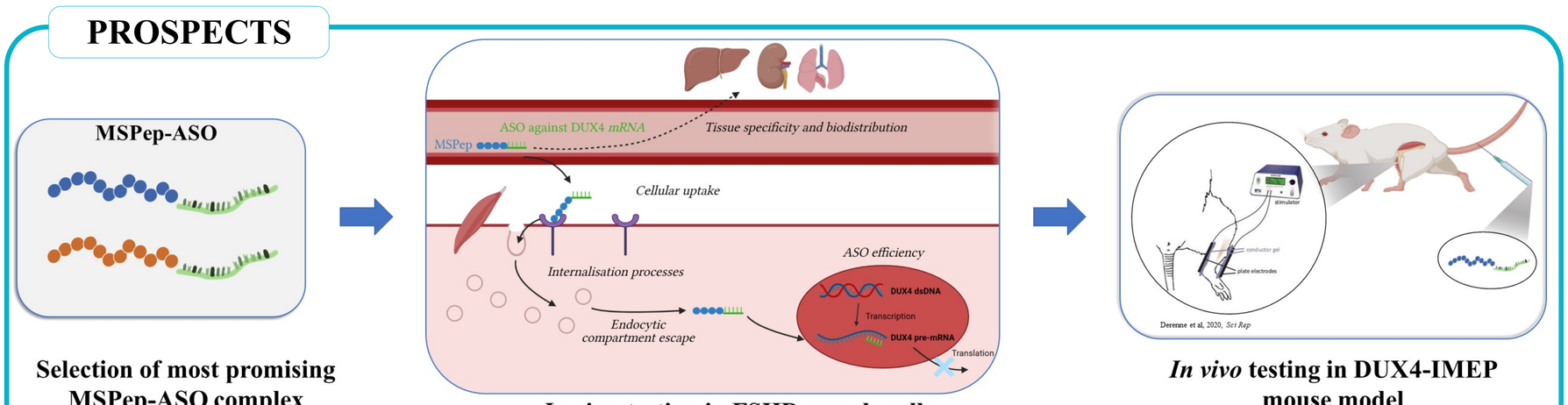
MSPep1 from screening #1 binds preferentially with myotubes as compared to hepatocytes and renal cells. MSPep1 also co-localizes with early endosomes suggesting an efficient internalization.

Concerning MSPeps 3-5 selected from screening #2, we observed their efficient endocytosis in myotubes and in non-muscle cells transfected with a plasmid encoding MMP but not in non-transfected cells used as negative controls.

Ongoing analyses aim to investigate MSPep endocytotic pathways and to confirm their skeletal muscle specificity.

The most promising MSpeps will then be complexed with ASO and tested *in vitro* and *in vivo*.

PROSPECTS



Selection of most promising MSPep-ASO complex

***In vitro* testing in FSHD muscle cells**

***In vivo* testing in DUX4-IMEP mouse model**

ACKNOWLEDGMENT

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