Selection of peptides for a muscle-targeted delivery of ASOs directed against DUX4 *mRNAs* through complementary approaches *in silico*, *in vitro* and *in vivo*.

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INTRODUCTION

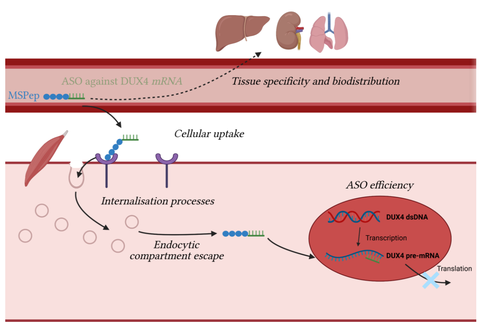
Facio-**S**capulo-**H**umeral **D**ystrophy is a hereditary myopathy that involves (epi)genetic components leading to the aberrant activation in skeletal muscle of ***DUX4***, a gene normally only expressed in germline and early embryogenesis.

GOAL OF THE STUDY

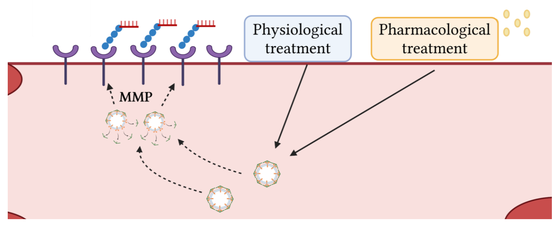
In a therapeutic approach for FSHD, AntiSense Oligonucleotides (**ASOs**) have been developed against the 3'UTR of *DUX4* mRNAs. Their use is currently limited because of their restricted tissue delivery, lack of tissue selectivity and rapid clearance by the liver and kidneys. Our strategy consists in coupling the ASOs with muscle-specific peptides (**MSPeps**) in the aim to improve ASO uptake into skeletal muscles.

AIMS

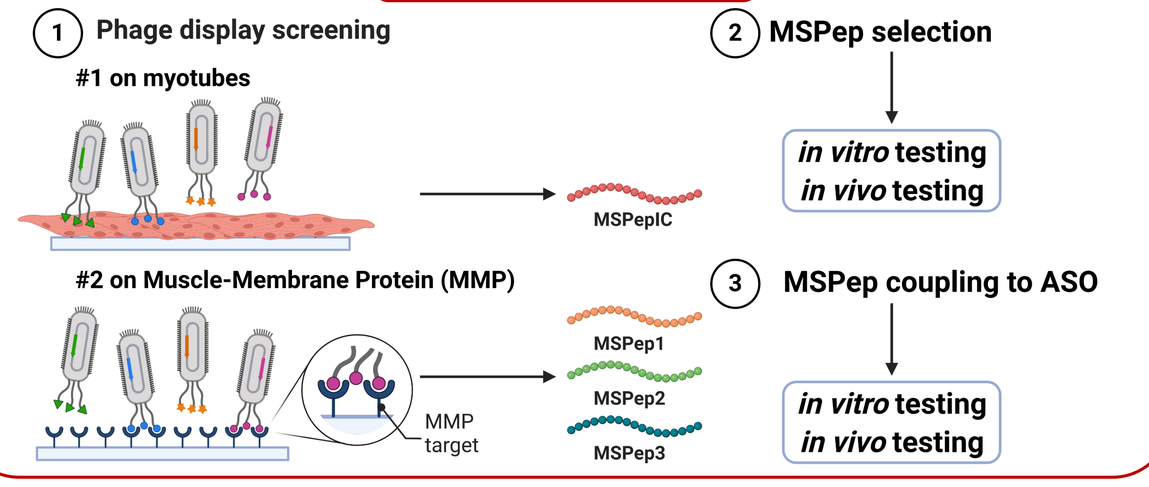
AIM#1. To decipher the biological properties of MSPep-DUX4-ASO



AIM#2. To ensure and improve MSPep1-3 endocytosis in FSHD muscle cells



METHOD



IN VITRO RESULTS

IN DIFFERENTIATED MUSCLE CELLS

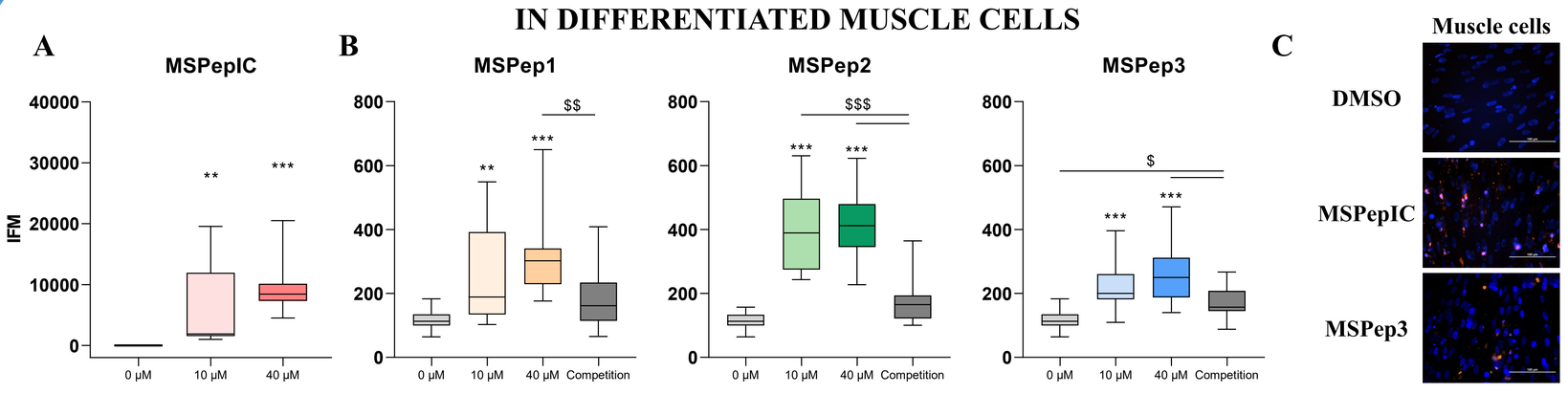


Figure 1: Internalization assays with rhodamine-MSPeps in muscle cells. A. Rhodamine-MSPepIC (0, 10, 40 µM) **internalization in human myotubes** (54-6 healthy controls). MSPepIC were added to culture medium of human myotubes during 2h at 37°C. Cells were then washed twice with PBS and stained with Hoescht 33342 (Invitrogen) for 5 minutes at 37°C. After two washes with PBS, cells were then fixed with PFA 4% for 10 minutes, washed and mounted with a glass slide before imaging with a fluorescent microscope. Intensity of fluorescence measured (IFM) represented as BoxPlots. One Way ANOVA followed by Dunn's Method; **: p<0.01, ***: p<0.001, vs 0 µM, n=2 (two technical duplicates on 2 independent cultures, 5 fields replicate). **B Rhodamine-MSPep1-2-3** (0, 10, 40 µM) **internalization in human myotubes (16Ubic).** Experiments were performed as described above. For binding competition, peptides (20µM) were pre-incubated with MMP (40µM). IFM represented as BoxPlots. One Way ANOVA followed by Dunn's Method, **: p<0.01, ***: p<0.001, vs 0 µM; $: p<0.05, $$: p<0.01, $$$: p<0.001, as indicated, n=2 (two technical duplicates on 2 independent cultures, 5 fields replicate). **C.** Representative images of MSPepIC and MSPep3 in human myotubes.

IN NON MUSCLE CELLS

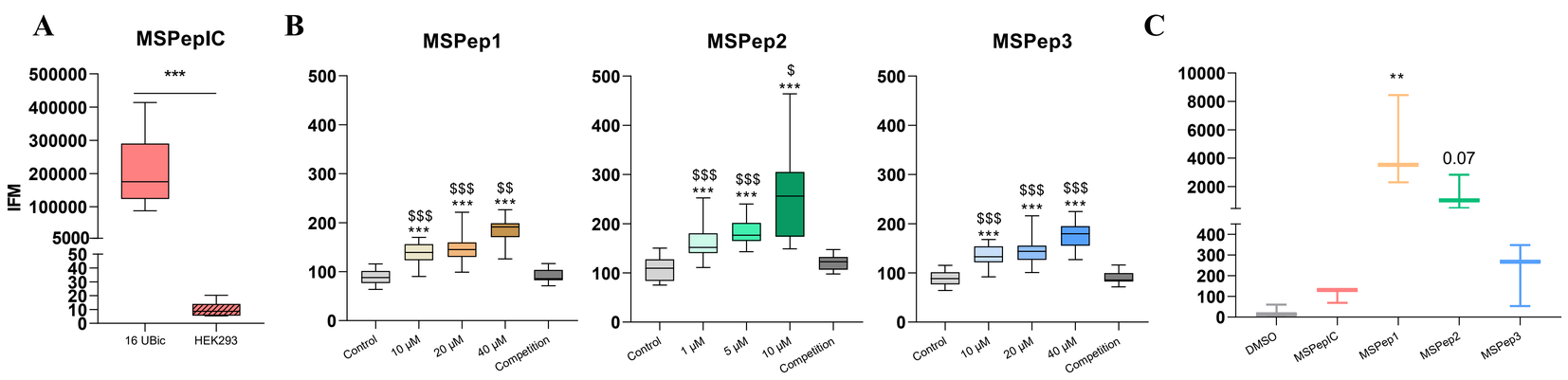


Figure 2: : Internalization assays with rhodamine-MSPeps in human embryonic renal cells (HEK293) and human hepatoblastoma cells (HepG2).

A. Rhodamine-MSPepIC (40µM) **internalization in human myotubes** (16Ubic) **and human embryonic renal cells** (HEK293). The experiments were performed as described above. IFMs are represented as BoxPlots. Mann-Whitney Rank Sum Test; ***: p<0.001n , n=2 (two technical duplicates on 2 independent cultures, 4 fields replicate). **B. Rhodamine-MSPep1-2-3** (40µM) **internalization in HEK293.** HEK293 renal cells were transfected or not with a MMP expression vector (used as positive control), and 24h later, the rhodamine-MSPeps were added for 2h at 37°C in the cell culture medium. For binding competition, peptides (10µM) were preincubated with MMP (20µM). IFMs are represented as BoxPlots. One Way ANOVA followed by Dunn's Method, ***: p<0.001 vs control (not transfected), $$: p<0.01 and $$$: p<0.001 vs competition , n=2 (two technical duplicates on 2 independent cultures, 5 fields replicate). **C. Rhodamine-MSPepIC-1-2-3** (40µM) **internalization in HepG2.** The experiments were performed as described above. IFMs are represented as BoxPlots. One Way ANOVA followed by Dunn's Method; **: p<0.001 versus DMSO, n=3 (two technical duplicates on 3 independent cultures, 5 fields per replicate).

IN SILICO RESULTS

A

	MSPepIC	MSPep1	MSPep2	MSPep3
pI	6,75	10	7,1	5,57
½ live (hours)	1,1	5,5	3,5	4,4
Aliphatic index (mol%)	65	73,33	32,5	98,33
Amino acid %				
Non polar (%)	33,5	33,3	33,3	75
Polar (%)	66,5	66,7	66,7	25
- Acidic (%) - Basic (%) - Non-ionisable (%)	- 16,7 - 24,9 - 24,9	- 0 - 58,4 - 8,3	- 8,3 - 41,7 - 16,7	- 0 - 0 - 25

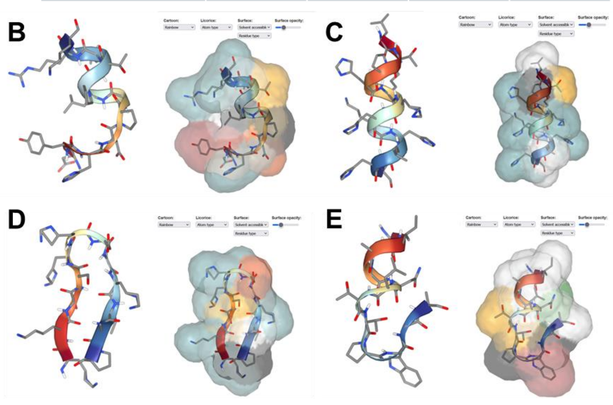
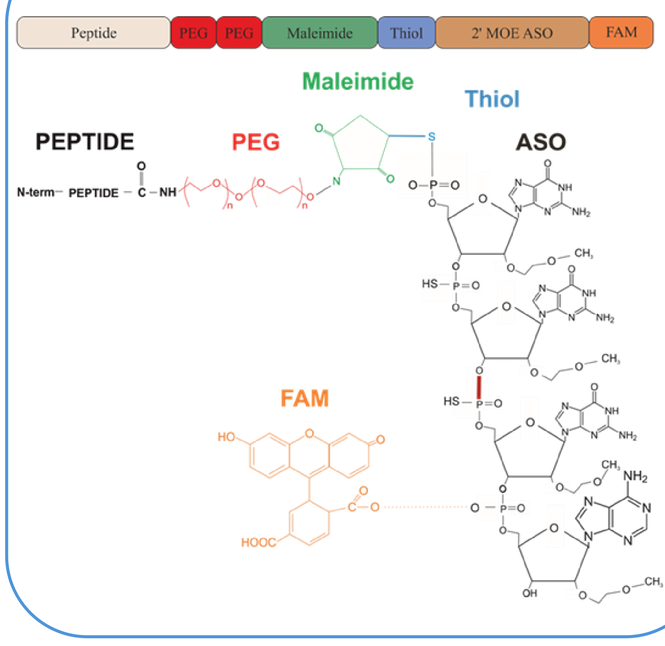
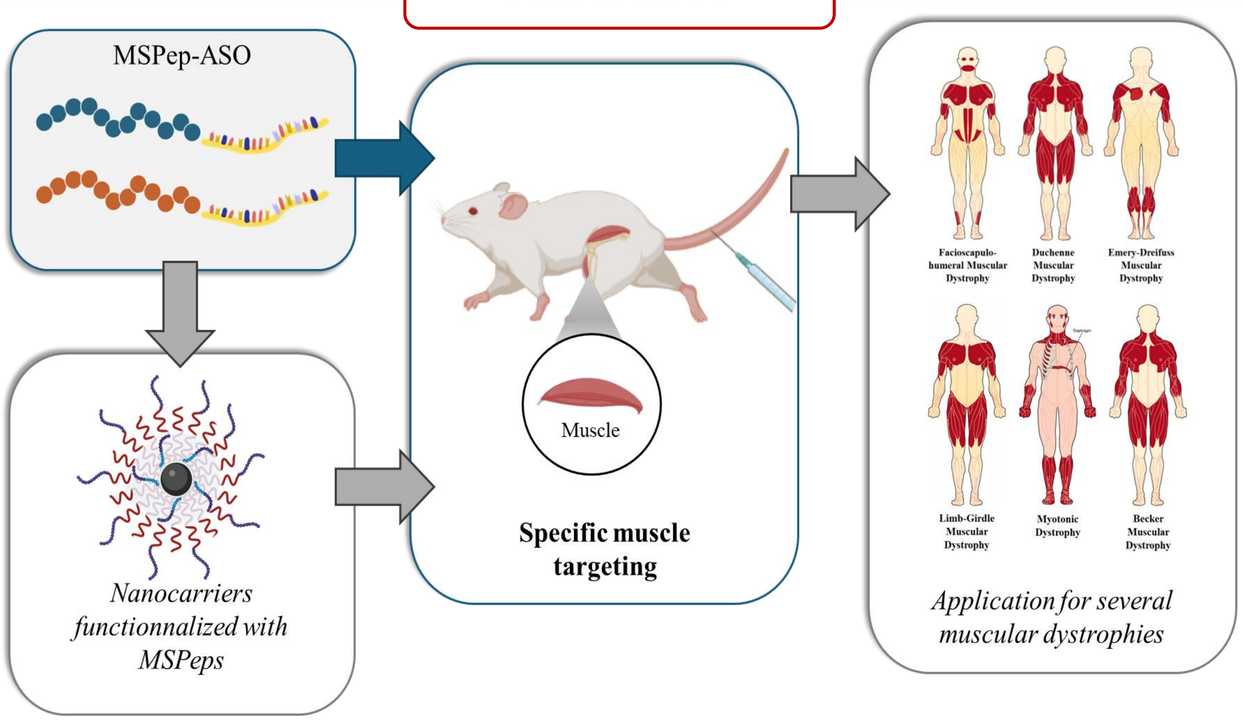


Figure 3. Biochemical characteristics of MSPeps and their tertiary structure. A. Biochemical characteristics were determined by using the ExPASy Software. **B-E.** Tertiary structure were determined by using the bioinformatic platform "RPBS" (https://mobyle.rpbs.univ-paris-diderot.fr/cgi-bin/portal.py). **(B)** MSPepIC. **(C)** MSPep1. **(D)** MSPep2. **(E)** MSPep3.

MSPep-ASO DESIGN



PROSPECTS



CONCLUSION

In **myotubes,** MSPepIC and MSPep1-2-3 are all internalization at the doses of 10 and 40µM.

In **renal cells,** MSPepIC is not internalized. MSPep-1-2-3 are not internalized by renal cells with no MMP expression but internalized when renal cells are transfected with a MMP expression plasmid

In **hepatocytes,** MSPep1 is internalized at a bigger extend than MSPepIC and MSPep2-3, likely due to its tertiary structure.The uptake of MSPepIC and MSPep3 in hepatocytes is limited and clearly lower than in myotubes.

Based on those analysis, MSPepIC and MSPep3 will be complexed with ASO.

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