Allele Selective and splice-switching FUS targeted antisense oligonucleotide therapeutic development for ALS

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Introduction

FUS is involved in important processess including gene regulation, DNA damage repair and in cellular responses to stress. Pathogenic FUS variants are associated with aggressive and early onset ALS. Antisense oligonucleotides (AOs) are nucleic acid analogues that are designed to bind to a target RNA and modulate gene expression through various mechanisms. A range of chemical modifications, including alternative nucleobase, sugar and linkage modifications are routinely used in AO design and have been essential to the success of AO based medicines. This work aims to improve on the AO therapeutic that is currently undergoing clinical trial in FUS-ALS patients by utilising different therapeutic strategies and chemistries.

Allele Selective Therapeutic Strategy

- FUS-ALS is a dominant disease making allele selective FUS reduction ideal.
- As over 50 pathogenic variants in FUS have been identified, we have targeted gapmer antisense oligonucleotides (AOs) to two common variants (CVs) in FUS for which are large proportion of people are heterozygous.
- Gapmers are AOs composed of a central core of DNA that is flanked by chemically modified ribonucleotides that protect the AO from degradation by nucleases. When a gapmer binds its target, the DNA:RNA duplex formed between the target RNA and the central DNA core acts as a substrate for the endogenous enzyme RNase H.

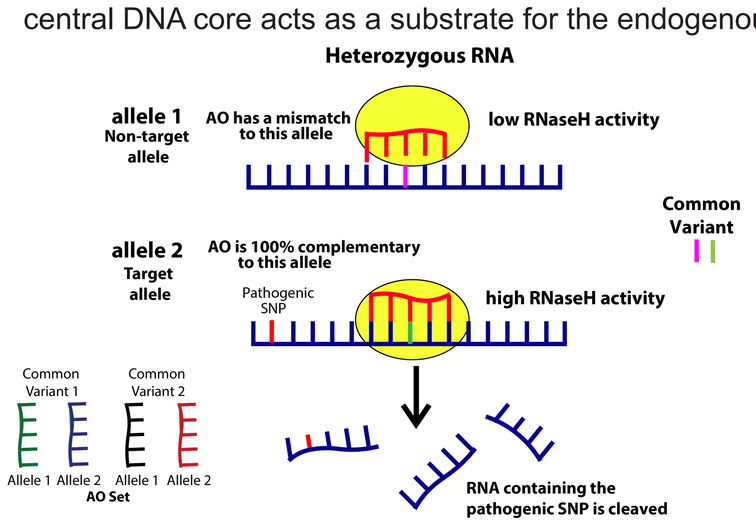


Figure 1: Schematic of allele selective therapeutic strategy. An AO is selected for the patient that is perfectly complementary to one allele at the site of a heterozygous common variant that is on the same allele as their pathogenic variant. This leads to transcripts from this allele being selectively degraded by RNase H.

Splice-switching therapeutic strategy

- Some FUS-ALS patients will not be heterozygous at the common variants targeted by our allele selective AOs.
- We have also developed a splice-switching AO that utilises the phosphorodiamidate morpholino oligomer (PMO) chemisrty.
- The FUS PMO reduces FUS expression by inducing exclusion of exon 7 from FUS mRNA, leading to a premature termination codon and nonsense mediated decay of FUS transcripts.

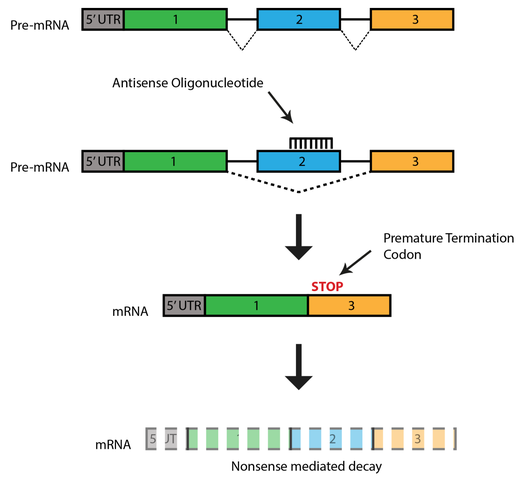


Figure 2: Schematic of exon skipping strategy. The AO binds to the pre-mRNA at a location that alters how the exon is recognised by the splicing machinery, leading to the targeted exon being excluded from the mRNA (exon skipping). This changes the reading frame of the transcript leading to a premature termination codon. Transcripts harbouring premature termination codons are degraded via the nonsense mediated decay pathway, an RNA surveillance mechanism that operates in the cytoplasm.

Antisense chemistries

- The current FUS therapeutic, Ulefnersen, is a gapmer with phosphorothioate (PS) linkage modifications and 2' methoxyethyl (2'-MOE) sugar modifications in the wings.
- Phosphorodiamidate morpholino oligomers (PMOs) have the five-carbon sugar rings substituted with 6-carbon synthetic morpholine rings that are joined via phosphorodiamidate linkages. PMOs exhibit enhanced nuclease resistance and safety due to reduced protein binding and immune activation. PMOs are not readily synthesised as chimeras with DNA bases so are not compatible with RNase H activity.
- Thiophosphoramidate morpholino oligomers (TMOs) are a hybrid oligonucleotide analogue that has 6-carbon synthetic morpholine rings that are joined via PS linkages. TMOs can be synthesised as chimeras with DNA bases.
- We found that the TMO modification reduced the formation of toxic structured nuclear inclusions/aggregates that are associated with PS containing AOs in comparison to 2'-MOE modified AOs and to an Ulefnersen mimic (Fig. 4).

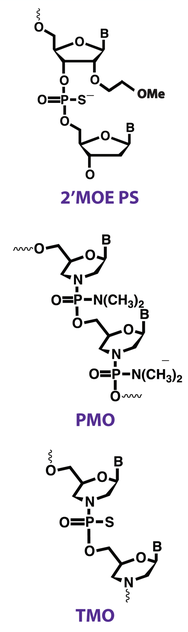


Figure 3: Antisense chemical structures

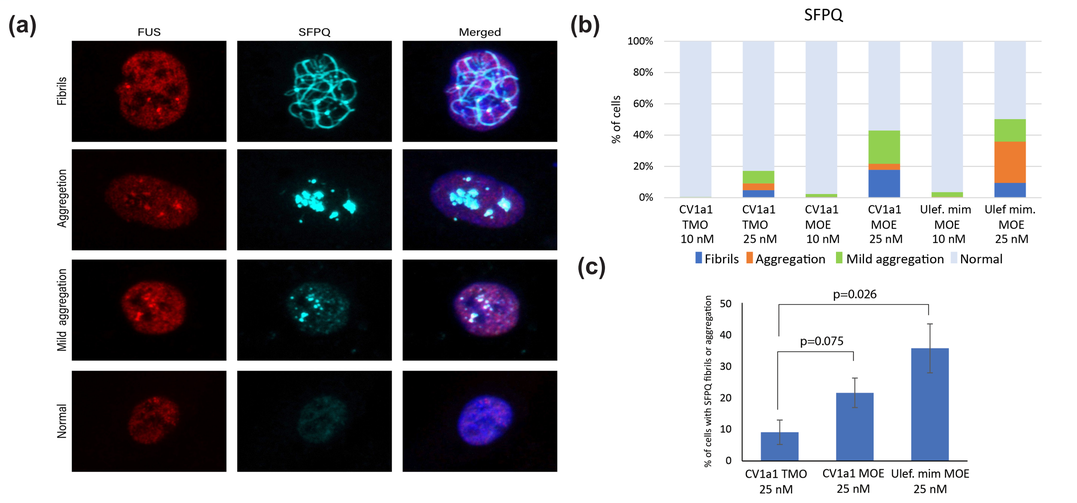
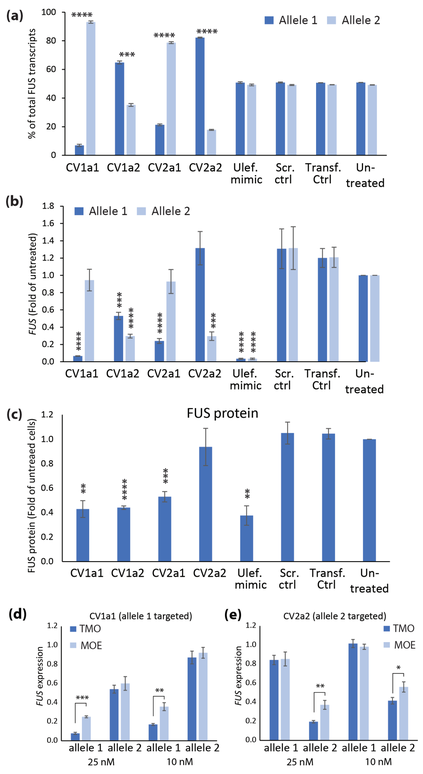


Figure 4: (a) Representative images of FUS and SFPQ immunostaining in human fibroblasts showing SFPQ fibrils and aggregation. (b) The mean percentage of human fibroblasts displaying SFPQ fibrils, prominent aggregation or mild aggregation 24 hours after transfection with a FUS targeted AO gapmer with TMO or MOE modified wings or an Ulefnersen mimic at 25 nM. (n=5). (c) Mean percentage of cells with prominent SFPQ aggregation or fibrils. 200 cells counted per experiment (n=5).

Allele selective TMO Results

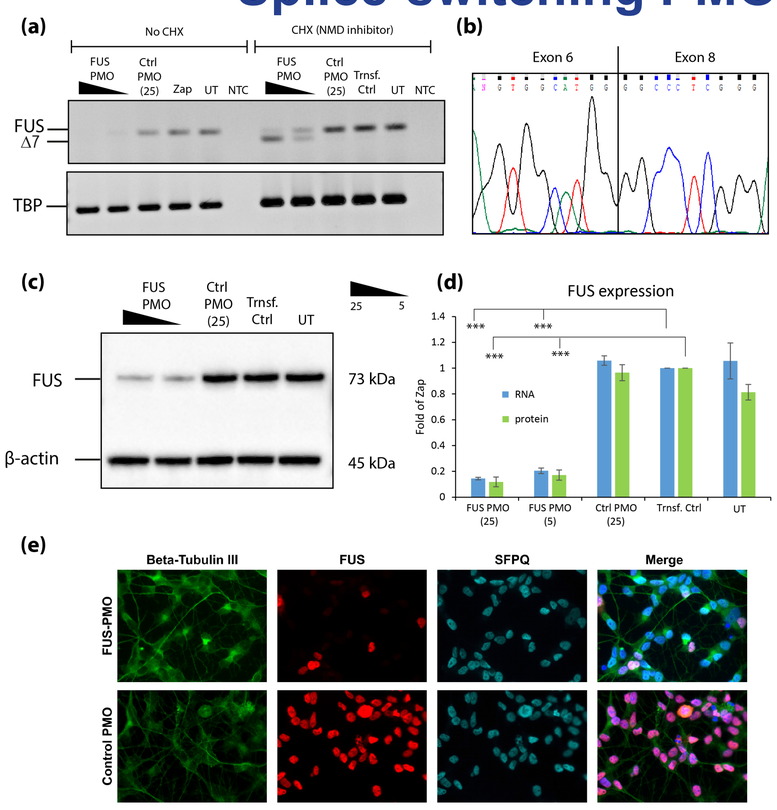


At 10 nM, the CV1a1 TMO (targeted to allele 1) reduced expression of allele 1 to 6.6% of control levels ($p<0.001$) with expression of allele 2 remaining at control levels after 5 days ($p=0.70$) (Figure 5b).

Greater allele selectivity was achieved using gapmers with TMO modified wings than with other chemical modifications including 2' methoxy-ethyl (MOE) (Figure 5d,e).

Figure 5: Human fibroblasts heterozygous at common FUS variants were transfected with AOs and RNA and protein analysed after five days. (a) Percentage of FUS transcripts of each allele measured by ddPCR after transfection with TMOs. (b) FUS mRNA expression presented as fold-change compared to levels in untreated cells normalized to levels of housekeeping gene TBP. (c) FUS protein levels measured by Western blot (normalized to total protein) expressed as fold-change compared to levels in untreated cells. (d) Comparison of MOE and TMO modified gapmer targeted to allele 1 (3 days). (e) Comparison of MOE and TMO modified gapmer targeted to allele 2 (3 days). Error bars represent standard error of the mean. (n = 3). * $p < 0.1$, ** $p < 0.05$, *** $p < 0.01$, **** $p < 0.001$.

Splice-switching PMO Results



The FUS PMO induces exclusion of exon 7 from the mature FUS transcript (Fig. 6a, b), leading to a shift in the reading frame and nonsense mediated decay (Fig. 6a), reducing FUS protein expression (Fig. 6c,d,e).

The PMO was tested in neuron-like SH-SY5Y cells, confirming that it is highly effective at suppressing FUS protein levels in human cells in vitro, with FUS reduced up to 88%.

Figure 6: Evaluation of FUS targeted PMO in neuronal-like human SH-SY5Y cells. (a) Cells transfected using electroporation, 25 and 5 μM (concentration in the tip during electroporation) and collected after five days. (a) RT-PCR of *FUS* transcript with and without the addition of nonsense mediated decay inhibitor, cycloheximide (50 μg/ml) for the final 6 hours of incubation. (b) Sanger sequencing chromatogram of product extracted from bottom band of (a) showing *FUS* transcript with exon 7 excluded. (c) Western blot showing FUS expression. (d) FUS mRNA levels (qPCR) and protein levels (Western blots) (n=3). *** indicates $p < 0.01$. (e) FUS and SFPQ staining in differentiated SH-SY5Y cells 11 days after transfection with FUS-PMO or a control PMO (25 μM) counterstained with Hoechst (blue) for nuclei detection (in merged image). Images were taken using the 40 x objective.

Conclusion

An allele selective TMOs or splice-switching PMO could be a promising therapeutic option for FUS-ALS patients. Future work will involve testing in patient iPSC-derived motor neurons and in animal models to demonstrate efficacy and the effects on FUS gain and loss of function.

Acknowledgements

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Black Swan PHARMACEUTICALS

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