Cystinosis Research Foundation Report - March 2015
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Background

Cystinosis is caused by mutations in the cystinosin gene (*CTNS*). The most common mutation worldwide is a 57kb large deletion mutation (LDM); however, the majority of patients in Quebec carry the W138X point mutation that encodes a premature stop codon. In order to develop an autologous cell-based therapy, we have devised a strategy to correct different types of cystinotic mutations with targeted nuclease technology using W138X and the LDM as models of different mutation types. Targeted nucleases mediate genome editing by creation of double-stranded breaks in DNA and subsequent repair by endogenous DNA repair machinery. Repair can occur via either non-homologous end joining (NHEJ), which involves error-prone repair without a template, or homology-directed repair (HDR), in which a donor template with homology to the targeted site is used to dictate insertion of the desired sequence.

Another strategy to treat stop codon mutations like W138X is the use of nonsense suppressor drugs, which increase read-through of stop codons during translation to produce functional protein. Geneticin (G418) is one such drug and there are others available (including Ataluren) that are well tolerated and show potential for use in human therapies.

Objectives

1. Correction of the W138X mutation in fibroblasts from a cystinotic patient using targeted nucleases and a single-stranded donor template.
2. Gene replacement in fibroblasts from a cystinotic patient carrying the 57 kb LDM using targeted nucleases for the *AAVS1* locus and a donor plasmid containing the correct *CTNS* sequence.
3. Testing of nonsense suppressor drugs on fibroblast cells from patients homozygous for the W138X stop codon mutation.
4. New *Ctns*$^{-/-}$ mouse models.

Objective 1. CRISPR/Cas9 correction of W138X mutation

Progress. To correct the W138X mutation, *CTNS*$^{W138X/W138X}$ fibroblasts from a cystinotic patient were transfected with a CRISPR/Cas9 nuclease targeting the mutation site and a single-stranded oligonucleotide donor (ssODN) containing the wildtype TGG codon plus 40 or 100 nucleotides of flanking homology. Clonal expansion was performed, however, no clones were identified with the corrected base pair. The

experiment is being repeated with reduced levels of CRISPR/Cas9 nuclease in an effort to improve viability of edited cells.

Future work. Clonal expansion of the repeat experiment and genotyping for corrected base pair by RFLP and sequencing.

Objective 2. Gene replacement of *CTNS* at the *AAVS1* locus.

Progress. Progress on this objective is pending success of objective 1. To replace the *CTNS* gene in patient-derived fibroblasts carrying the LDM, CRISPR/Cas9 and a donor plasmid will be used to target insertion of a *CTNS* expression construct at the *AAVS1* locus. *AAVS1* is a 'safe harbor' for gene insertion with consistent levels of gene expression and relatively few positional effects. HDR will be mediated by a donor plasmid containing the correct *CTNS* sequence and flanking homology to the targeted *AAVS1* site. We have generated a Pcmv-*CTNS*-His expression construct which will be used for gene replacement.

Future Work. The donor plasmid will be constructed by inserting Pcmv-*CTNS-His* into a commercial plasmid with 1.6 kb of flanking homology to target insertion at the *AAVS1* locus. We will then transfect $CTNS^{LDM/LDM}$ patient fibroblasts with the donor plasmid and a CRISPR designed to cleave *AAVS1*. Clonal cell lines will be obtained by dilution plating and assessed both by sequencing of the insert borders and by Western blot for CTNS and His.

Objective 3. Testing of nonsense suppressors on $CTNS^{W138X/W138X}$ human fibroblasts

Progress. In addition to previous data showing that G418 reduces cellular cystine and increases CTNS protein levels, we now have evidence of an effect on *CTNS* mRNA levels. We see an increase in steady-state *CTNS* mRNA after treatment with G418 for 24 hours. Our results suggest that G418 does indeed reduce nonsense-mediated mRNA decay, the process by which the premature STOP codon causes disease. Normal fibroblasts do not show a difference in *CTNS* mRNA levels with or without the addition of G418.

Future Work. Positive results from our experiments with G418 have lead to an agreement to acquire a new nonsense suppressor drug from Eloxx Pharmaceuticals. This drug was developed from G418 but modified to improve the nonsense suppressor effect and abolish toxicity, making it a more clinically viable treatment option. We are testing this drug on human fibroblasts harboring the W138X mutation and will then perform *in vivo* tests using our mouse model with a premature STOP mutation in *Ctns*.

Objective 4. New $Ctns^{-/-}$ mouse models

4.a) Generation of $Ctns^{-/-}$ CD1 mice containing a premature stop codon mutation for testing of nonsense suppressor drugs

To test the effect of nonsense suppressors *in vivo*, we have generated a mouse line with a premature stop codon in exon 8 of *Ctns* using ZFNs and a donor oligo to introduce the mutation. Preliminary assessment of cystinotic phenotype has been performed and, at 6-6.5 months of age, these mice show elevated cystine in the kidney tissue, swan neck deformity and elevated glucose in the urine when compared to wildtype CD1 mice.

Future work. Further characterization will be performed on mice between 6-9 months of age, as this is the period over which we would hope to see improvements in mice subjected to therapies. Once characterized, nonsense suppressor drugs (including G418 and the Eloxx compound) will be tested *in vivo* in these mice and assessment will be made for any improvement in tissue cystine levels, *Ctns* mRNA levels, and glucosuria.

4.b) Generation of *Ctns*$^{-/-}$ NOD/SCID mice for testing of human renal progenitor-like cells with potential for use in cell therapy of cystinosis.
In order to test human cells *in vivo* without the need for an immunosuppressant drug we have generated an immunodeficient mouse model with a mutation in *Ctns*. A CRISPR/Cas9 nuclease was used to target exon 8 of *Ctns* in conjunction with a donor oligo designed to introduce a premature STOP codon. Homozygotes have been identified and a breeding colony has been established.

Future work. Colony expansion is on-going and characterization will be performed on mice at 6-9 months of age, in order to have a better understanding of the disease in our model. We will test tissue cystine levels, *Ctns* mRNA levels, and glucosuria.