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Cystinosis Research Foundation Report - April 2014
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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 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.

Objective 1. ZFN-mediated correction of W138X mutation

Progress. To correct the W138X mutation, $CTNS^{W138X/W138X}$ fibroblasts from a cystinotic patient were transfected with zinc finger nucleases (ZFNs) targeting the mutation site and a single-stranded oligonucleotide donor (ssODN) containing the wildtype TGG codon plus 42 or 62 nucleotides of flanking homology. Cel-I assay of the resulting cell population showed the presence of a wildtype sequence in 4.6% of alleles for oligo 42 (500 pmol) and 3.9% of alleles for oligo 62 (300 pmol). Low viability of cells transfected with the ZFN pair has thus far prevented us from obtaining a clonal cell line. However, cells

transfected with the ssODN alone did not show a viability problem and we therefore predict that off-target effects of the ZFNs are the cause.

Future work. Due to cell viability issues when using ZFNS, we have adopted CRISPR/Cas9 as our targeted nuclease of choice for future experiments. We have designed a CRISPR and oligos with 40 or 100 bp of homology to target W138X and the correction experiment will be repeated. Clonal cell lines will be obtained by dilution plating and assessed for correction by sequencing of *CTNS* exon 7.

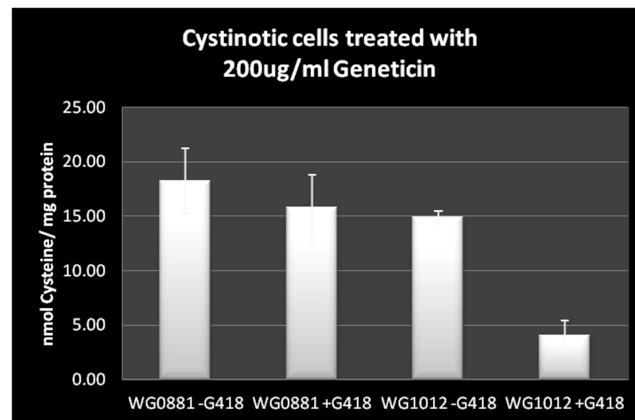
Objective 2. Gene replacement of *CTNS* at the *AAVS1* locus.

Progress. 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. Results from HPLC analysis of patient-derived fibroblasts treated with geneticin (G418) show a significant decrease in cystine levels in geneticin(G418)-treated $CTNS^{W138X/W138X}$ cells (WG1012) that is not seen in $CTNS^{LDM/LDM}$ cells (WG0881), indicating that the effect is specifically due to stop codon read-through.



As another tool to test the effect of nonsense suppressor drugs on cystinotic cells, we have introduced the W138X mutation into our pCMV-*CTNS*-His construct. This will enhance our ability to detect an effect at the protein level by detection of the His tag.

Future work. Fibroblasts from *CTNS*$^{W138X/W138X}$ patients will be transfected with the pCMV-CTNS(W138X)-His plasmid then treated with nonsense suppressor drugs. Read-through will be assessed by measurement of Cystine levels by HPLC and by Western blot detection of both CTNS and the His tag.

Additional Progress

Generation of *Ctns*$^{-/-}$ CD1 mice containing a premature stop codon mutation

To test the effect of nonsense suppressors *in vivo*, we have generated a homozygous mouse line with a premature stop codon in exon 8 of *Ctns*. ZFNs and a donor oligo were used to target *Ctns* in the pronucleus of CD1 mice, resulting in 2 heterozygotes out of 7 viable pups. These 2 females were backcrossed to CD1 mice and the F1 recovered and inbred to produce homozygotes. A total of 6 homozygotes were produced in the F2 (2 males, 4 females) and will be used to establish a colony.

Future work. Nonsense suppressor drugs, including Geneticin and Ataluren, will be tested *in vivo* in our CD1 *Ctns*$^{-/-}$ mouse line. Cystine levels will be measured in various tissues by HPLC and CTNS protein will be detected by Western blot.

Progress on generation of *Ctns*$^{-/-}$ immunodeficient mice

In order to test human cells *in vivo* without the need for an immunosuppressant drug, we aim to generate an immunodeficient cystinotic mouse model. ZFNs were initially used in attempts to generate *Ctns*$^{-/-}$ NOD-SCID mice, however, no viable pups were born from approximately 100 targeted pronuclear zygotes. We then used a different type of nuclease called CRISPR/Cas9 to target exon 8 of *Ctns* in conjunction with a donor oligo designed to introduce a premature STOP codon. NOD-SCID mouse embryos were targeted at the pronuclear stage and 60 embryos were implanted in pseudopregnant females resulting in 9 viable pups, 5 of which are heterozygous for the exon 8 stop mutation. The heterozygotes were backcrossed to NOD-SCID mice and the resulting litters are shown in the table below:

High dose CRISPR	**litters**	**genotype**
#1 male	sterile	
#2 female	1st litter 2nd litter	died yet to be genotyped
Low dose CRISPR		
#3 female	1st litter	1 heterozygous female, NHEJ allele in the founder
#4 female	1st litter	2 heterozygous females, NHEJ allele in the founder
#5 female	1st litter	3 heterozygous females, correct HDR allele (exon 8 stop)

Future work. Heterozygous females from the litter of female #3 will be bred in order to produce homozygotes, however, we require a heterozygous male to make a breeding pair.

iPSC training in the lab of Dr. Sharon Ricardo, Monash University, Melbourne, Australia.

Thanks to funds from the CRF, I was able to spend 2 weeks learning iPSC maintenance and manipulation techniques in the lab of Dr. Sharon Ricardo at Monash University, Melbourne. These protocols will be used in future experiments relating to generation of renal progenitor-like cells for stem cell therapy of cystinosis.