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Cystinosis Research Foundation Report - September 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 (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.

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 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. Resulting clones have been expanded and are currently undergoing genotyping to identify a cell population with the corrected base pair. Only 8 have been genotyped so far and these 8 were all uncorrected.

Future work. Genotyping of clones is on-going. If no clone of interest is identified, the experiment will be repeated with a reduced level of CRISPR plasmid introduced to improve cell viability of edited clones (and thus, increase chances of successful expansion).

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.

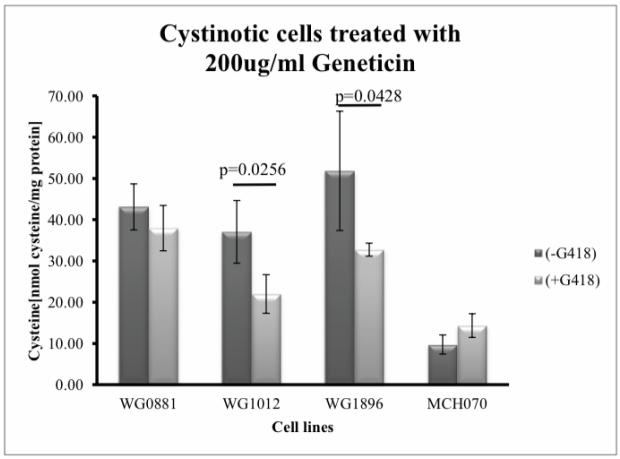
Progress on generation of $Ctns^{-/-}$ immunodeficient mice

In order to test human cells *in vivo* without the need for an immunosuppressant drug, we aim to generate an immunodeficient cystinotic mouse model. 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 mice are being aged for assessment of the cystinotic phenotype.

Future work. Characterization of cystinosis in the mouse line.

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

Progress. Results from HPLC analysis of patient-derived fibroblasts treated with G418 show a significant decrease in cystine levels in 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. Two homozygous W138X fibroblasts lines, WG1012 and WG1896, displayed reductions of ~60% in pathologic excess cystine (subtracted from the MCH070 normal fibroblast line). No significant change was observed in the homozygous 57kb deletion line, WG0881.



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 and pCMV-*CTNS*-DsRed constructs. These constructs were transfected into a cystinotic cell line harboring 57kb deletion mutations and treated with geneticin. Both the His tag and DsRed could be detected after G418 treatment, but not in controls without G418.

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

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. Homozygotes have been identified and mice are being aged for assessment of the cystinotic phenotype.

Future work. After characterisation of cystinosis in the mice, nonsense suppressor drugs, including G418 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.