Cystinosis Gene Repair: Progress Report #3 (Sep 2011 – Jun 2012)

Ciaran LEE (Research Fellow), Patrick HARRISON (PI/Mentor); Martina Scallan (Co-PI)

Department of Physiology and Microbiology, University College Cork, Ireland

Introduction

The overall aim of this fellowship is to develop a robust method for *ex vivo* manipulation of the *CTNS* gene in intact cells by using genome repair rather than cDNA addition. It will provide precisely engineered cell lines which will enhance the efforts of many research groups to further understand the mechanism by which *CTNS* mutations and deletions cause cystinosis, and may also benefit cell and gene-based therapies in development for treating cystinosis. Below is a summary of progress to date and refinements to the strategy based on advances in gene repair technology in the context of the four specific goals of the project.

Specific Goal 1: development of ≥13 ZFN pairs/donor sequences to repair mutations/deletions

To date, 14 pairs of *CTNS*-specific ZFN pairs have been designed and tested in collaboration with Katrin Kaschig (a PhD student in the PI's lab funded by Cystinosis Ireland) using three separate design methods. These ZFNs were tested using both *in vitro* and *ex vivo* assays. Several ZFN pairs could cleave synthetic target sequences *in vitro* (Figure 1) but none of these were active *ex vivo* (Figure 2).

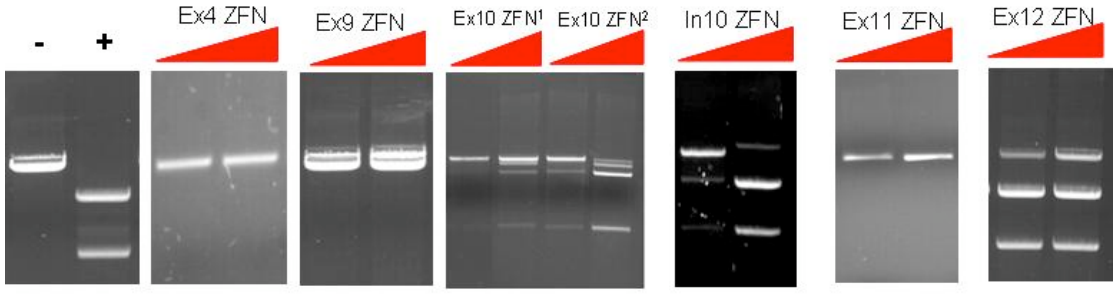

Figure 1: *In vitro* ZFN assays. All ZFN assays used 1µg of target plasmid linearized with *Xba*I. Positive control (+) plasmids cut with *Xba*I and *Xho*I. Target DNA was incubated with 10ng or 100ng of ZFN protein (1:1 and 10:1 ZFN:DNA molar ratios). Cleavage was observed with ZFN pairs targeting *CTNS* exon 10, intron 10 and exon 12.

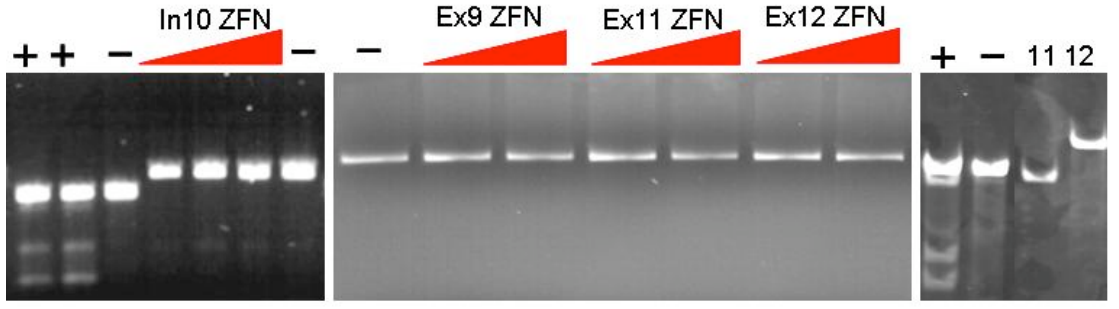

Figure 2: *Ex vivo* ZFN assays. Positive controls (+) are *CFTR* ZFNs designed in PI's lab (Lee et al., 2012). *Ex vivo* assays were carried out in ZFN-treated epithelial cells. Successful gene targeting results in Indel formation via NHEJ which is detected by CEL-I nuclease assay (Lee et al., 2012). No significant ZFN activity was observed suggesting that CTNS targeting has not occurred, or is below the detection limit of the assay (<1%). The third panel shows data from an alternative assay shows effective gene targeting of + control, but no activity with CTNS exon 11 or exon 12 ZFNs.

A disparity between efficient *in vitro* activity and minimal *ex vivo* activity of ZFNs has been widely reported in the last two years and as a consequence, most labs have now switched to an improved DNA cleavage/repair strategy using TALE nucleases (TALENs; Mussolino and Cathomen, 2012). We have designed 10 different TALENs to target three regions of *CTNS*: intron 3, exon 4, and intron 11. The first of these *CTNS*-specific TALENs will be tested with mini-gene repair constructs outlined below.

Specific Goal 2: optimize delivery of ZFNs and donor sequence

We have optimized delivery of ZFNs and donor sequences using another gene in our lab (Lee et al., 2012). For cystinosis, our original aim was to use the same strategy of using individual donor sequences for each exon. However, a more efficient method to repair multiple exons using mini-gene donors has recently been used in a mouse model of hemophilia (Li et al., 2011). We have designed mini-gene donor constructs for use with TALENs targeted to intron 3 and intron 11 (see Figure 3). These constructs contain flanking homology arms either side of CTNS partial cDNA sequences. The cDNA sequences contain the appropriate splicing signals to allow recombination of cDNA exons 4-12 (using intron 3 TALENs) or exon 12 LKG (using intron 11 TALENs) into mutant CTNS alleles. These donor constructs also contain the EGFP reporter gene for enrichment via FACS and a self-cleaving T2A peptide to remove EGFP from CTNS during translation (Figure 3). Together, this combination of TALENs and mini-gene donors should allow correction of virtually all known CTNS mutations.

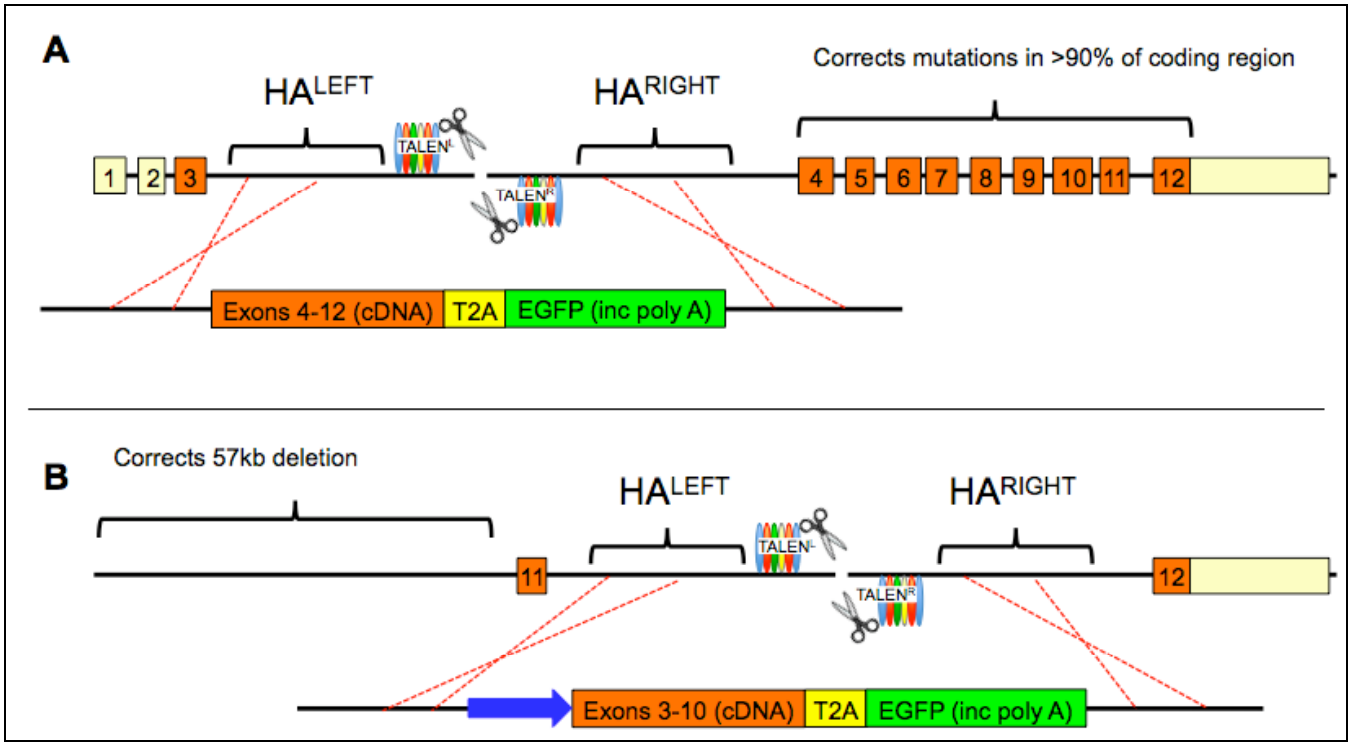

Figure 3) A – intron 3 mini-gene construct (not to scale): This repair construct has been designed for use with an intron 3 specific TALEN pair to repair any mutation in exons 4-12. Left and right intron 3 homology arms (HA) target the exon 4-12 and EGFP construct to the site of nuclease cleavage. A self cleaving T2A peptide sequence between CTNS cDNA and EGFP allows for both to be expressed as separate proteins from a single ORF. A lack of promoter in the targeting construct ensures EGFP expression occurs only following targeted integration and expression from the native CTNS promoter allowing targeted cells to be enriched via FACS. **B – intron 11 mini-gene construct:** This repair construct has been designed for use with an intron 11 specific TALENs to repair the 57 kb deletion, or any promoter/exon 3 mutations that cannot be repaired by the intron 3 mini-gene construct. Left and right intron 11 HA target the promoter/exon 3-11 and EGFP construct to the site of nuclease cleavage.

The intron 11 TALENs will also be used to generate cell lines expressing only one of the CTNS isoforms (WT or LKG) to further evaluate the physiological role of the C-terminal region of the cystinosin protein (see Taranta et al., 2012; not shown in Figure 3).

Specific Goal 3: evaluate off-target events

Whilst some off-target binding of ZFNs can occur, the use of TALENs should significantly lower the incidence of such events (Mussolino and Cathomen, 2012). Nonetheless, assays to measure off-target cleavage will be used to determine the safety profile of each TALEN pair.

Specific Goal 4: fully exploit potential of ZFN/TALEN gene repair through active collaborations

Preliminary results were presented in poster format at the European and Society for Gene and Cell Therapy conference in October 2011. We received positive feedback and detailed technical advice from leading ZFN and TALEN research groups which has been incorporated into our work in the last 8 months. The gene repair system from this work can be used to create cell lines to study role of cystinosin-LKG isoform and mechanism of stem cell engraftment. Minor modifications of the constructs to remove the T2A sequence would allow cystinosin-EGFP fusion proteins to be expressed under the control of the endogenous promoter; proteins modified in this way give a much more accurate understanding of trafficking processes (see Doyon et al., 2011). Finally, this technology can be readily adapted to permanently correct HSCs from cystinosis individuals; this would restore cystinosin function under the control of its endogenous promoter (Bellomo et al., 2010).

References

Bellomo F, Corallini S, Pastore A, Palma A, Laurenzi C, Emma F, Taranta A. (2010). Modulation of CTNS gene expression by intracellular thiols. Free Radic Biol Med. 48:865-72. PMID: 20079424

Doyon JB, Zeitler B, Cheng J, Cheng AT, Cherone JM, Santiago Y, Lee AH, Vo TD, Doyon Y, Miller JC, Paschon DE, Zhang L, Rebar EJ, Gregory PD, Urnov FD, Drubin DG (2011). Rapid and efficient clathrin-mediated endocytosis revealed in genome-edited mammalian cells. Nat Cell Biol. 13:331-7. PMID: 21297641

Lee, CM, Flynn, R, Hollywood, JA, Scallan, MF and Harrison, PT (2012). Correction of the ΔF508 mutation in the CFTR gene by zinc finger nuclease homology-directed repair**.** BioResearch Open Access *in press*. PMCID: *in progress*

Mussolino C and Cathomen T (2012). TALE nucleases: tailored genome engineering made easy. Curr Opin Biotechnol. 2012 Feb 17. [Epub ahead of print]. PMID: 22342754

Taranta A, Petrini S, Citti A, Boldrini R, Corallini S, Bellomo F, Levtchenko E, Emma F. (2012). Distribution of cystinosin-LKG in human tissues. Histochem Cell Biol. 2012 Apr 29. [Epub ahead of print] PMID: 22544350