

## A proteomic investigation of cystinotic cells and the effects of cysteamine treatment.

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### CRN Project Update August 2012

#### Introduction

Since the last update to the CRN in February 2012, the major focus of the project has remained on the acquisition and analysis of whole cell data for cystinotic and control cells (obtained from UZ Leuven with permission from Prof. Dr. E. Levchenko). Using the results from the initial experiments for cell doubling time and labelling the proteome of cystinotic cells, we have successfully labelled cystinotic fibroblasts with heavy (<sup>13</sup>C) arginine and lysine. The initial SILAC results for cystinotic and control fibroblasts were presented as a poster at the 7<sup>th</sup> International Cystinosis Congress in Paris (June 2012).

#### Labelling the proteome of cystinotic cells

Knowledge of the growth curve and doubling time of the cells allows the time needed for complete incorporation of the heavy label, in this case, <sup>13</sup>C-arginine and lysine, to be calculated. Complete incorporation is imperative for the accurate quantitative comparison of the two populations (cystinotic vs control). In previous experiments, the doubling time of cystinotic fibroblasts was investigated by counting the number of cells in the population over a period of 72 hours and was calculated as 35.5 hours. Cystinotic fibroblasts were grown for 8 doubling times (approximately 3 weeks) in heavy labelled (<sup>13</sup>C-arginine and lysine, R6K6) cell culture medium. A sample of cells was taken prior to transferring the population into the heavy medium and then again at the end of the incorporation period. These cells were lysed using sonication and the proteins from them separated by 1D SDS-PAGE (fig 1). To assess the incorporation of the heavy label, identical bands from both the unlabelled and labelled samples were excised and digested using trypsin prior to mass spectrometric analysis of the resulting peptides. The heavy and light peptides are identical apart from a mass difference which corresponds to the mass difference between the heavy and light isotopes (6Da, in this case), as shown in figure 2. The light peptides, corresponding to any unlabelled peptides are barely detected at 534-536 m/z therefore showing the complete incorporation of <sup>13</sup>C in the labelled cystinotic fibroblasts.

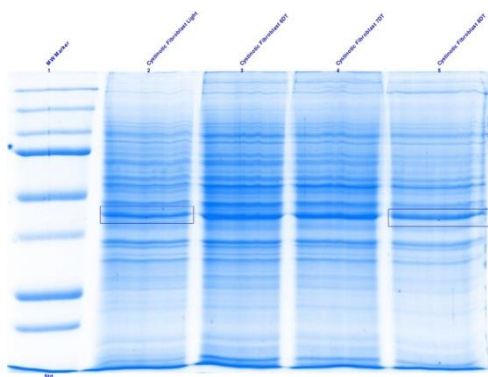


Figure 1 Protein lysates from cystinotic fibroblasts were separated by 1D SDS-PAGE with coomassie blue staining. Annotated bands were excised and digested for mass spectrometry analysis.

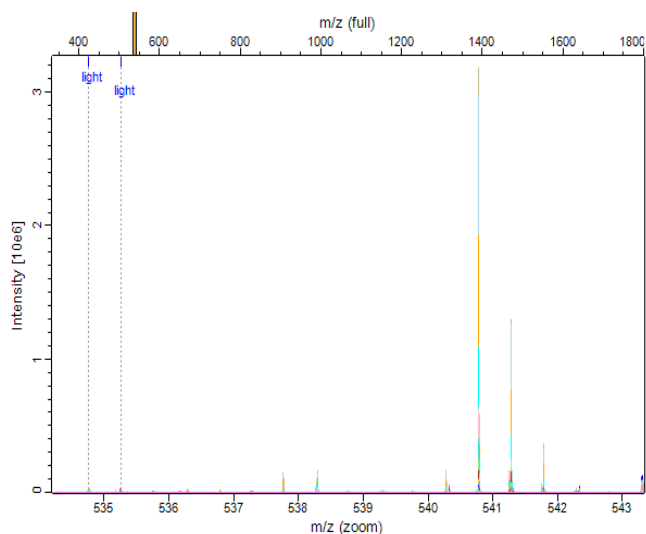
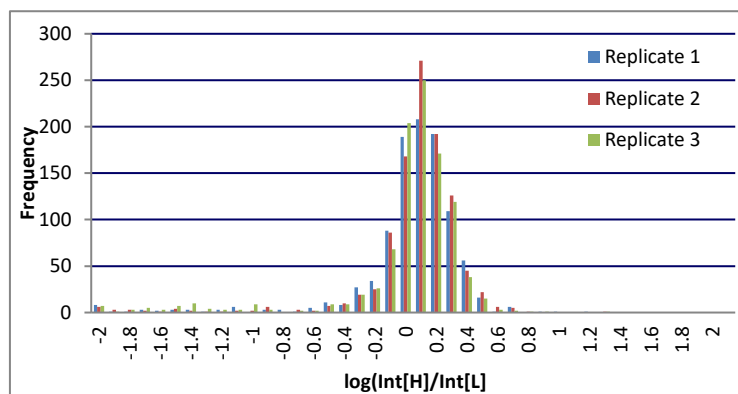


Figure 2 Mass spectrum of a peptide derived from cystinotic fibroblasts. The heavy peaks at m/z 540-542 correspond to the heavy labelled peptide. The light peptides, expected at m/z 534-536, are barely detected.

## *Quantitative comparison of the proteome of SILAC labelled cystinotic fibroblasts and the corresponding unlabelled control cells.*

Following the complete incorporation of the heavy label into the proteome of cystinotic fibroblasts, SILAC analysis was carried out using a 1:1 mixture of proteins from labelled cystinotic and unlabelled control fibroblasts. The samples were analysed by mass spectrometry and database searching carried



**Figure 3** Distribution of the ratios of the 1383 identified proteins with quantifiable heavy/light ratios presented on a log<sub>10</sub> scale from 3 biological replicates.

out using the MaxQuant programme. The ratio of the intensity of the heavy and light isotopes was used to determine proteins that are up or down-regulated in cystinotic cells in comparison to control fibroblasts. In total, 1383 proteins were identified with 821 being quantified in all 3 biological replicates (figure 3). In a separate experiment, unlabelled cystinotic and control fibroblasts were analysed separately and the protein identities obtained using the global proteome machine (GPM). KEGG pathway analysis of these results showed that unique proteins found in control fibroblasts were involved in vesicle transport, core metabolic and amino acid metabolism pathways; these key pathways may be expected to be affected in cystinosis patients and appeared to be significantly down-regulated, or even absent, in cystinotic cells.

Further data analysis of these results is on-going with the view of validating the results through the use of biological and technical replicates of all samples. Using the results from the SILAC experiment, proteins that have been quantified in 2 out of 3 and 3 out of 3 biological replicates are being identified and statistical analysis performed to assess the significance and confidence of these results. Pathway analysis will also be performed on these results and the data collated to form the first publication of the project. Over the next 6 months, the experiments will be repeated using cystinotic and control proximal tubule epithelial cells to highlight any similarities or differences between the two cell lines. These experiments provide a baseline for the protein expression in cystinotic cells in comparison to well-validated control (non-cystinotic) cells. Although not the major focus of the project, an additional output from the results of these experiments could be the identification of alternative analytical biomarkers for the detection and subsequent diagnosis of cystinosis.

If funded, the final 12 months of the project (1<sup>st</sup> Feb 2013 – 31<sup>st</sup> Jan 2014) will build on these initial data and, using the same methodology, we will investigate the effects of cysteamine on cystinotic cells to identify which pathways are improved or modified by the treatment and whether any affected pathways remain unchanged. Data and pathway analysis of this investigation could also lead to the identification of potential new treatment targets.

**Update on collaborator Prof Achim Treumann:** we are delighted to report that Prof Treumann has found permanent employment at the nearby University of Newcastle upon Tyne and will remain an active collaborator on the project.

## *Financial Update*

As of 23<sup>rd</sup> August, the balance stands at £32,404 (> \$51,000); there remain several purchases outstanding, including an expensive specialist piece of analytical software from Non-Linear Dynamics, the purchase of which we have delayed pending an update (just released). This software provides great versatility and the ability to convert mass spectrometric data sets into different formats for more detailed analysis; we have training organised with this new software for September 2012. There also remains the cost of mass spectrometry analysis of the cystinotic vs control experiments outlined above, which will cost around £6,000-8,000 (ca. \$9,500-\$12,500). We anticipate that the balance will cover the outstanding commitments and SILAC experiments comparing cystinotic and control cells, as outlined above, which will support the work over the next 6 months. To complete the comparison of untreated with cysteamine-treated cystinotic cells, we request that the additional funds for the 3<sup>rd</sup> year of research, as detailed in the original proposal, of £39,697 (\$62,760) should be granted.