The role of the human COBW gene family in cellular zinc metabolism

Background: I It has been indicated through genome analysis that up to 10% of all human genes may encode for proteins that bind zinc. The important and diverse role of zinc in biology makes it important to understand the way in which zincis controlled within the cell. In this context, it is unkown how zinc is transported around the cell and delivered to the appropriate metalloproteins (contrasting with copper, about which much is known about intracellular chaperones). The COBW gene family was brought to the interest of Dianne Ford's laboratory through a genome wide search of upstream promoter sequences, looking for a regulatory DNA sequence motif first seen within the zinc-regulated *SLC30A5* gene.

Hypothesis and Aims: Our hypothesis was that COBW genes play a central role in zinc metabolism within the cell and may have a zinc chaperone function. The aims of the project were to investigate the expression of COBW, how it is regulated and to gain an insight into the cellular function of the COBW protein product.
The specific objectives were:

- to investigate the localisation of the COBW protein product when expressed from a transgene as a FLAG-tagged fusion (to allow detection using an anti-FLAG antibody) in Chinese Hamster Ovary (CHO) cells, including the effect of different extracellular zinc concentration;
- to measure the response of the COBW protein to zinc by western blotting, using the same experimental model;
- to identify the tissue-expression profile of COBW by RT-PCR analysis of a panel of mRNAs from a wide range of human tissues.

Description of work and results:
CHO cells were grown on glass cover slips and then transfected (Genejammer; Stratagene) with a recombinant plasmid based on the pCMV6-entry vector from Origene, with the open reading frame for human COBW3 inserted into the multiple cloning site. This vector uses the mammalian CMV promoter to achieve high-level gene expression and incorporates a FLAG epitope tag onto the C terminus of the product. Cells were treated 24 h after transfection with different concentrations of extracellular zinc (3 – 100 μM). The transfected cells were fixed using a methanol:acetone mix, incubated with anti-FLAG antibody conjugated to FITC and then mounted in medium including DAPI, to stain nuclei. Cells were viewed using fluorescence microscopy. Localisation of the FITC signal, revealing the localisation of COBW protein, was throughout the cell including, in most cases, the nucleus, but appeared to be excluded from nucleoli (Figure 1). In a small proportion of cells there was some evidence for nuclear exclusion (Figure 1). There was no obvious effect of zinc treatment on this distribution of the recombinant COBW protein.

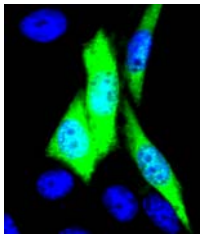

Figure 1. Expression of FLAG-tagged recombinant human COBW in transfected CHO cells. Anti-FLAG immunofluorescence is shown as green as nuclei are stained blue with DAPI. Note the apparent exclusion of COBW from the nucleus of the cell at the top right of the image.

The same experimental system was used to study the effect of different concentrations of extracellular zinc on the level of expression of the COBW protein, but in this case cells were cultured on plastic, in six-well plates, rather than on glass cover slips. After transfection and zinc treatment (24 h), cells were washed with and scraped into PBS and a cell lysate was prepared. Equal quantities (10 μg) of protein from cells treated at the different zinc concentrations were separated by SDS-PAGE , blotted onto PDVF membrane and probed using an anti-FLAG primary antibody, which was detected using and HRP-conjugated secondary antibody. Signals were then revealed using ECL chemiluminescent reagent (Amersham Life Sciences) and captured on X-ray film. Typical results are shown as Figure 2. A specific band of the expected molecular weight (~45 kDa) was detected in extract from cells transfected with the COBW construct (but not in extract from negative control cells, transfected with vector only) and this band increased in intensity, indicating an increase in COBW protein levels, in response to zinc (100 μM); this response was confirmed in three independent experiments.
Finally reverse transcription PCR was performed on RNA from different human tissues (Total Human RNA Tissue Panel; Ambion) to examine if the COBW3 protein is expressed throughout the body or in specific tissues. Another student in the laboratory completed the PCR step (since initial results showed contamination in negative control reactions) after the end of the current project. The results are shown in Figure 3 and reveal that the COBW3 protein is expressed in all tissues tested, possibly at realtively low levels in the spleen and relatively high levels in the testes.

Discussion: The work carried out indicates that COBW is expressed widely, across a diverse range of human tissues, consistent with it playing a fundamental role in cell function; a zinc chaperone function would ne one such role, but the data as yet in no way support this view. The notably high level of expression measured in testes may relate to the essentiality of zinc for the development of testes and sperm and for the health of sperm. The broad pattern of

localisation at the cellular level gives little indication about the likely function of the protein, but this distribution would not be inconsistent with COBW playing a fundamental role intracellular zinc handling, including as a zinc chaperone. Regulation of COBW expression at the protein level in response to zinc is consistent with the protein being involved in some function that is important with respect to zinc transport or homeostasis, but more rigorous and detailed investigation is required to gain information about the specific nature of any such role.

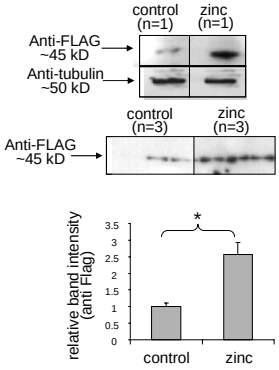

Figure 2. Regulation by zinc of COBW protein expressed in CHO cells. CHO cells were transfected with a construct from which COBW was expressed as a FLAG-tagged fusion (FLAG on the C-terminus) and lysate was analysed by western blotting, using an anti-FLAG antibody, 24 h after addition of either control medium (3 µM Zn; labelled "control") or high-zinc medium (100 µM Zn, added as $ZnCl_2$; labelled "zinc"). The blots show two independent experiments. The blot show in the upper panel was re-probed with anti-tubulin antibody to confirm equal loading and transfer. The bar chart shows the result of densitometric quantification of the blots shown. Data are normalised to control and are expressed as mean ± SEM; $P<0.05$ by Student's unpaired t-test.

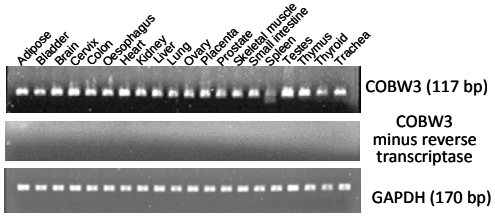

Figure 3. Detection of COBW expression by RT-PCR in different human tissues (Total Human RNA Tissue Panel; Ambion). The COBW PCR product and its size are shown in the top panel. The middle panel shows the same analysis without the addition of reverse transcriptase and confirms absence of any DNA contamination. The bottom panel shows the result of PCR amplification of the same reverse transcription reactions using primers specific to GAPHD, included as a positive control for successful cDNA svnthesis.

Future directions of the Project: As part of the current study, some preliminary inconclusive data were obtained concerning effects of divalent metals other than zinc (cobalt, nickel and copper (II)) on COBW expression, detected by western blotting, indicating that the response of COBW to metals may not be zinc-specific. These experiments should be repeated to identify with confidence which other divalent metals regulate COBW expression. Additional approaches to investigate possible effects of divalent metals, including zinc, on COBW localisation could involve cell fractionation then detection by western blotting. The mechanism of COBW regulation in response to zinc should be investigated in further detail, for example by use of quantitative RT-PCR to establish if there is any regulation at the level of mRNA and through use of COBW promoter-reporter constructs to investigate transcriptional regulation in response to zinc; such experiments are, in fact, already underway in the laboratory. Identification of proteins that interact with COBW, for example by co-immunoprecipitation, could provide further clues as to the function of COBW.

Value of the studentship: To me the studentship has been invaluable. It has allowed me to experience how a research lab runs and is run, and given me an appreciation that what is done in taught practical classes is designed to work within the time constraints of a degree program. I have been able to perform techniques and use equipment that I have either only heard of, or used only once in a very structured environment. Mammalian cell culture has been an exciting and new technique that I would not have had the chance to experience had I not been given this studentship. I have been involved in designing my own experiments and structuring them to ensure that time is not wasted, while receiving advice and knowledge from those in the lab around me. Finally this experience will become a hugely important part of my CV, for jobs inside and outside the scientific community. I would like to thank the Biochemical Society for allowing me to take the time to do this studentship and to Dianne Ford and her lab for the project, help and support given to me.

Value of the studentship to the host laboratory: Lewis was a fantastic addition to the research group over the summer and through his hard work derived some key preliminary data on this topic. These data will support applications for full project funding for this work, which is of high priority in our overall research programme on zinc. Lewis's work made an essential contribution to work presented to the Physiological Society at the Epithelia and Membrane Transport Themed Meeting of the Physiological Society, September 2009, Newcastle University, and Lewis is included as an author on the peer-reviewed, published abstract (Tyson J, van der Hagen EAE, Coneyworth LJ, Bell LS, Hadfield JN, Ford D (2009). Responses of the COBW domain-containing genes to zinc in human Caco-2 intestinal epithelial cells and effects of zinc on the COBW domain-containing protein. Journal of Physiology (abstract in press)). I thank the Biochemical Society for the award.