EXPRESSION OF ENDOPLASMIC RETICULUM OXIDOREDUCTASES (EROS) AND THEIR ROLE IN THE GI TRACT

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CHAPTER 6

DISCUSSION

The present study focussed on the characterisation of Ero1α and Ero1β in oesophageal cell tissues, which is an interesting environment to study Eros *in vivo*. The GI environment experiences a number of physiological perturbations, including alteration of pH and exposure to bile acids. Cells present in these tissues may have special biochemical adaptations to function in these fluctuating conditions. The results presented in this work lead to the following main conclusions:

1) Ero1α is constitutively expressed in OE33 adenocarcinoma cells at a higher level than in OE21 squamous carcinoma cells. Ero1α in OE33cells is mainly present in the inactive OX2 form. Ero1β is not expressed in either of these types. Ero1α, Ero1β and PDI do not undergo changes in expression or oxidation state as result of treatment at different pH or with bile acids in OE21 or OE33. There is no difference in expression of ERp57 between OE21 and OE33, though ERp72 is possibly more highly expressed in OE33.

2) Ero1β was purified as a HIS-tagged protein, which remains stable for up to 24 hours either at 4 °C or 24 °C. The recombinant Ero1β -HIS protein was unreactive with reduced TRX, but shows potential reactivity with reduced PDI.

3) Screening a panel of 61 potential anti-Ero1α/Ero1β supernatants did not identify any unambiguous candidates, but did identify a number of supernatants that could be investigated further for re-testing against Ero1 conformers.

6.1 Differential Ero1α expression between OE21 and OE33

Immunohistochemical studies have shown tissue-specific expression patterns for Ero1α and Ero1β (Dias-Gunasekara *et al.*, 2005). Ero1α transcripts are prominent in the oesophagus (Pagani *et al*., 2000), while Ero1β is found in the enzyme producing chief cells of the stomach, and in pancreatic islet cells, along with high expression of the ER

chaperones PDI, ERp57 and BiP (Dias-Gunasekara *et al.*, 2005, and NCBI microarray ID: GDS426/85466_at/ERO1LB/*Homo sapiens*; Yanai *et al.*, 2005). In section 3.2.2 , Ero1α expression was shown to be consistently higher in OE33 cells (Figure 3.4A), with no additional increased expression of either PDI (Figure 3.4B) or ER chaperones ERp57 and HSP90 (Figure 3.4C and E). There may be a slightly higher expression of ERp72 in OE33 compared to HeLa and OE21 (Figure 3.4D). ERp72 is highly expressed in the stomach, and could also be expressed at a higher level in metaplastic Barrett's oesophagus tissue. The nature of the possible difference in expression of ERp72 observed in OE33 oesophageal adenocarcinoma cells warrants further work, although the exact function of ERp72 remains unknown. However, ERp72 does belong to an intra-ER multi-chaperone complex (Meunier *et al.*, 2002). ERp72 has five thioredoxin-like domains, the only known member like this in the PDI family. Three of these thioredoxin-like domains are catalytic. The protein surface is highly charged, and lacks the hydrophobic binding pocket present in PDI and Pdi1p (Kozlov *et al.*, 2009, Kozlov *et al.*, 2010). As ERp72 could function in a different manner to PDI, it's possible interaction with Ero1α (or Ero1β) could be explored further with pulse-chase and co-immunoprecipitation experiments, in vitro protein interaction studies or employing *in silico* binding models based on solved crystal structures (Masui *et al.*, 2011). Such work would help to evaluate the importance and significance of the observed expression difference of ERp72 in OE33 compared to OE21 and HeLa. Recently, it has been suggested that Barrett's metaplasia may originate from competitive interactions between two cell lineages, present in the oesophagus (Wang *et al.*, 2011b). Human Barrett's tissue lacks the tumour suppressor p63. The authors showed that p63-null mice lack squamous epithelium, and develop intestine-like metaplasia during embryogenesis. This suggested that Barrett's metaplasia could develop in an environment free from chemical insults and chronic inflammation. The

murine embryonic metaplasia had gene expression profiles similar to those found in human Barrett's oesophagus. The murine metaplasia was tracked through embryogenesis, and shown to persist in adult mice. Discrete clusters of cells, with similar gene expression profiles to the mouse metaplasia model were found in the wildtype murine squamocolumnar junction and could also be found in human tissue at this same location. The authors suggest that chronic reflux, typical in Barrett's patients, drives a positive selection pressure in favour of the growth of this extant metaplasia, from cells with their origin in remnants of embryogenesis. Although OE33 cells are derived from a patient with Barrett's adenocarcinoma, they may potentially represent a cell culture model of the growth and proliferation of this embryonic metaplasia in a patient with chronic reflux. The investigation of thiol-disulphide exchange mechanisms and the ER stress response in this cell type is worthy of investigation. The expression levels of Eros in the murine metaplasia model is unknown.

The high expression of Ero1α in OE33 was predominantly seen in the inactive OX2 form (Figure 3.4A).The reason for the difference in Ero1α expression remains unclear. The monomeric OX2 form is the stable native state of Ero1α in living cells (Benham *et al.*, 2000), and possesses an additional long-range disulphide absent from the OX1 form, accounting for its faster electrophoretic mobility on SDS-PAGE gels. Appenzeller-Herzog and Ellgaard demonstrated the molecular switch mechanism between the OX1 (active) and OX2 (inactive) forms. This was dependent on the availability of reduced PDI (Appenzeller-Herzog and Ellgaard, 2008). Mutagenesis and gel mobility studies showed that the long-range regulatory disulphide unique to the OX2 form of Ero1α was between Cys94-Cys131. This regulatory covalent bridge effectively blocks the first shuttle cysteine, Cys94. The authors suggested that the presence of reduced PDI disables Ero1α OX2 inactivation by Cys94-Cys131, and returns Ero1α to the OX1 state. Under oxidising conditions, PDI accumulates in an oxidised form, and Ero1α returns to

its fully oxidised OX2 state. OE33 cells may require an abundant source of Ero1α, to provide oxidising equivalents. This may be related to the need to produce disulphide-containing secretory proteins such as MHC class I molecules (Section X.X) and mucins shown in Figure 3.16 (Section 3.4), and as previously published (Mariette *et al*., 2004). The PDI homologue AGR2 (Mariette *et al*., 2004) has been shown to be associated with disulphide-rich mucins, which are in turn experience an expression increase as a result of bile acid treatment in OE33 cells (Park *et al.*, 2009). In the present work, AGR2 expression could not be profiled either by Western blot, or via immunofluorescence (Figure 3.17 and 3.19). Using RT-PCR, one could determine AGR2 expression at the message level, and confirm any difference between OE21 and OE33. AGR2 has only one functional cysteine (Persson *et al.*, 2005), so is unlikely to facilitate dithiol-disulphide exchange reactions. Instead, it may perform as an isomerase, and re-shuffle disulphide bonds in mucins. It is likely that oxidising equivalents are required in the folding of mucins, and it is possible that these could be provided by Eros. AGR2 is associated with adenocarciomas, and recently it has been shown that it can co-opt the Hippo and EGF signalling pathways, promoting tumour growth (Dong *et al.*, 2011). Ero1α and PDI can heterodimerise, as demonstrated by co-immunoprecipitation studies (Benham *et al.*, 2000). It would be interesting to see if Ero1α or Ero1β could co-precipitate with AGR2, a PDI family member, and if so, how this interaction takes place, using recombinant proteins. Until recently, the mechanism of selective oxidation of PDI by Ero1α, in preference to its family members, was unknown. Using *in silico* simulations and biochemical analysis, it was shown that a β-hairpin of Ero1α preferentially interacts with a pocket in the PDI **b'**-domain. This leads to the shuttling of disulphides from Ero1α, oxidising the C-terminal PDI **a'**-domain, then from oxidised PDI to its substrate. (Masui *et al.*, 2011). Dissociation of Ero1α from PDI occurs when PDI is oxidised. In this state, PDI was shown to have ten-fold

less affinity for Ero1α. When the Ero1α β-hairpin was abolished *in vivo*, oxidative folding of model proteins such as J-chain is substantially, but not completely, reduced, suggesting there are other minor determinants in Ero1α-PDI docking, yet to be determined. However, this hairpin was less important in the docking of PDI family member, ERp44, which dissociated twenty-fold faster than PDI when oxidised. Interestingly, a C-terminus ERp44 mutant inhibited the Ero1α-PDI oxidation reaction. This work suggests that there are a variety of mechanisms in place for Ero1α-PDI family selectivity (Masui *et al.*, 2011).

Previous surgical studies and a handful of microarray data accessible via pubmed give interesting but sometimes conflicting data about Eros in the GI tumour environment. In the present work, Ero1β was not expressed in OE21 or OE33 cells, and could not be induced either by altering the media to a lower pH, or treating with bile acids.

A study of the gastric mucosa in a rodent model pre-disposed for gastric carcinoids and treated with loxtidine for 8 weeks or 16 weeks, resulted in development of hyperplasia or neoplasia respectively (NCBI microarray ID: GDS576/97870_s_at/Ero1l/Mus musculus [unpublished]). This data shows an increase in Ero1α expression in either outcome, and an increase in Ero1β expression in neoplasia.

Studies of gastric tubular adenoma and carcinoma also gave unclear results as to gene expression differences for Ero1α and Ero1β, though Ero1β might be decreased in carcinoma (NCBI microarray ID: GDS1792/684/ERO1L/Homo sapiens; Lee *et al.*, 2005).

The study of OE21 and OE33 in this thesis showed no change in expression or oxidation state in Ero1α following pH treatment, and a low pH cell culture model of gastric reflux using the oesophageal cell line SKGT4, also did not show any noticeable trends in Ero1α or Ero1β mRNA expression over a 240 minute timecourse. Genes which were induced following low pH treatment included those involved in the

response to DNA damage (EGR1-4, ATF3) and in cell-cycle control (GADD34, GADD45, p57) (NCBI microarray ID: GDS1286/218498_s_at/ERO1L/Homo sapiens; Duggan *et al.*, 2006).

The precise role that Ero1α may play in gastric carcinomas is uncertain. One of the main problems in comparing cells from GI carcinomas, particularly adenocarcinomas, is the wide variety of heterogeneity seen between patients. As such, the difference in Ero1α expression in OE33 may not be indicative of all Barrett's cases or all adenocarcinomas, thus further work is required to compare the expression levels of Ero1α in more cell lines and patient samples.

It was recently shown that Ero1α regulates calcium fluxes in cells (Anelli *et al.*, 2011, Chin *et al.*, 2011). Overexpression of Ero1α leads to an increase in the passive efflux of calcium from the ER into mitochondria, via the mitochondrial membrane associated ER membranes (MAM). This pathway involves Ero1α, ERp44 and the inositol triphosphate receptor type 1, IP3R1. Ero1α accumulates at the ER-MAM junctions, and overexpression of Ero1α increased the efflux of calcium from the ER, lowering intra-ER calcium levels. The authors suggested that overexpression of Ero1α oxidises IP3R1, and removes the blocking of this receptor by ERp44. When Ero1α expression was silenced, the MAM pool of Ero1α decreased, fewer IP3R1 receptors were oxidised and a reduced mitochondrial calcium uptake was measured (Anelli *et al.*, 2011). Such redox changes in the cysteine residues of calcium channels in cardiac tissue have also been shown to be affected by Ero1α expression (Chin *et al.*, 2011). Decreased Ero1 expression in mice resulted in an attenuated cardiomyocyte response to adrenergic stimuli, and the absence of Ero1α was protective in murine surgical models of heart failure.

Intriguingly, this new role for Ero1α suggests it may be involved in calcium fluxes in oesophageal adenocarcinoma, particularly with reference to the cyclic AMP-response

element-binding protein (CREB)-NADPH oxidase (NOX5-s) pathway (Fu *et al.*, 2006). NOX5-s was shown to be expressed at a higher level in both SEG1-EA (oesophageal adenocarcinoma) cells and dysplastic Barrett's tissue, compared to squamous cells and non-dysplastic Barrett's tissue. Treatment at pH4 significantly raised intracellular calcium, and activated CREB; this was responsible for acid-induced expression of NOX5-S, which mediated the acid-induced production of H_2O_2. Whether this feeds into Ero1α and ER-MAM calcium would be worthy of further investigation. Clearly the overexpression of Ero1α in OE33 adenocarcinoma cells compared to OE21 squamous carcinoma cells merits further study with respect to the role of calcium.

6.2 Purification of Ero1β

Ero1β was first described in 2000, as a similar protein to Ero1α, that was upregulated as a consequence of the UPR (Pagani *et al.*, 2000). Ero1β is expressed in the chief cells of the stomach and in pancreas, whereas Ero1α expression is more universal (Dias-Gunasekara *et al.*, 2005).

The online database Ensembl suggested three alternate splice sequences for Ero1β, containing 16, 7 or 4 exons. It has not been shown previously if the 4 or 7 exon sequences are expressed at all. Here, the expression of the three alternate splice forms of Ero1β was tested in HeLa, OE21 and OE33 cells. Expression of full-length Ero1β can be seen in HeLa cells under certain conditions. At no time in these cells was Ero1β-7 or Ero1β-4 expression seen, either innately or as a consequence of ER stress or bile acid treatments (Figures 4.3-4.6).

An Ero1β-HIS protein was purified using nickel-agarose columns from an Ero1β-HIS-GST construct expressed in origami bacteria using the PGEX4T-3 vector, modified from a published method used to purify Ero1α (Baker *et al.*, 2008). Ero1β could only be purified when the GST tag was cleaved whilst the fusion protein was bound to GST

beads, and was twice purified either side of the GST step with a Ni-NTA column. This recombinant protein remained stable for up to 24 hours either at 4 °C or 24 °C (Figures 4.12-4.13). Wang *et al*. also produced recombinant Ero1β, although they used BL21(DE3) pLysS cells, and the pGEX4T-3 vector (Wang *et al*., 2011). Their bacterial pellets were lysed with a sodium phosphate buffer, which did not contain protease inhibitor or imadazole. The GST tag was cleaved using PreScission Protease (Amersham) rather than thrombin, as described in Chapter 4. Thrombin was unlikely to result in non-specific degradation, and this is supported by data shown in Chapter 4 (Figure 4.13).The tag remaining on the Wang et al fusion protein was an N-terminus GPLGS pentapeptide. It would be interesting to directly compare the catalytic activity of the recombinant Ero1β proteins resulting from these two methods.

6.3 Ero1β function

Purified Ero1β was tested in oxygen electrode assays, using the reaction between an Ero and reduced TRX to initially determine its catalytic properties. This technique has been used for yeast Ero1p (Sevier *et al.*, 2007) and human Ero1α (Baker *et al*., 2008). In a reaction with reduced TRX, Ero1β-HIS was unable to return TRX to the oxidised state, which suggested that it was a catalytically inactive protein, in comparison to Ero1p or Ero1α. However, in the recent paper by Wang *et al*, Ero1β was shown to oxidise reduced TRX with reaction kinetics similar to Ero1α (Wang *et al*., 2011). Furthermore, Ero1β was also capable of facilitating the re-folding of RNAse A in the presence of human PDI, working twice as efficiently as Ero1α as an oxidase. Mutagenesis studies showed that the C-terminal tail of PDI was not required for its interaction with Ero1β. The PDI **a'** domain mediates electron flow from PDI to Ero1β, and binding required the PDI **b'** and **a'** domains. Mutagenesis and gel migration studies showed that Ero1β has three long-range disulphides, Cys81–Cys390, Cys90–Cys130

and Cys100–Cys262. The authors concluded that their recombinant protein was more loosely regulated than Ero1α, and operated at high speed in RNase A and PDI assays, although further work is required to demonstrate this for Ero1β activity *in vivo*.

A further test of Ero1β-HIS activity was carried out in oxygen electrode recordings of Ero1β-HIS with 100μM reduced PDI, in the presence of GSH (Figure x?). In published data, when Ero1α was reacted with PDI and 10mM GSH, 160 mM oxygen was consumed, roughly equal to 80% oxidation of the PDI active sites. The maximal turnover of Ero1α was approximately three disulphides formed per molecule of Ero1α per minute, comparable to Ero1p (Sevier *et al.*, 2007). In this thesis, when Ero1β-HIS was reacted under the same conditions, there was consumption of oxygen over 44 minutes, which is comparable to data for Ero1α, purified under similar conditions (Baker *et al*., 2008). This data compares with that published with Wang, *et al*. 2011b. It can be inferred that Ero1β-HIS was able to bind to and engage in thiol-disulphide exchange with PDI via the **b'** and **a'** PDI domains.

The lack of activity of Ero1β-HIS with TRX suggested that TRX may not be a compatible substrate with Ero1β, unlike PDI. It was originally thought that this may be due to the differences in the redox active motifs between Ero1α and Ero1β, though this reason may not be the case because the recombinant Ero1β protein produced by Wang *et al*. was capable of fully reducing TRX in five minutes.

Another point worth noting is that the Ero1β protein produced in this study lacks glycans. Ero1β has four glycosylation sites, compared to Ero1α, which has two. The presence of glycans was discussed in the introduction; they are large bulky sugars, that affect the way a protein folds (Shental-Bechor and Levy, 2008, Shental-Bechor and Levy, 2009). One biophysical effect of glycans is to increase protein stability. Furthermore, large bulky glycans present on Ero1β may act naturally to block stable disulphide bond conformations in the protein. One possibility is that in the absence of

glycans, a stable non-native disulphide bond in Ero1β may result, rendering the protein less active.

In Ero1β it has been shown that the major regulatory bond is between Cys90-Cys131. In this oxidised state, the Cys90-Cys95 bond is eliminated, and the protein is inactive (Wang *et al*., 2011). A comparison of the regulatory bonds present in Ero1α and Ero1β is shown in Figure 6.1. Yet to be addressed is the functional role that the four N-glycans have in Ero1β, as the recombinant protein produced by Wang, *et al*., like that produced in the present study, would also have been produced without glycans.

The expression, substrate specificity and functional kinetics of human Eros remain an intriguing area of research. This thesis has explored the expression of Ero1α in cell culture models of oesophageal adenocarcinoma, and concluded that there is an expression difference between the squamous (OE21) and adenocarcinoma (OE33) cells tested. Ero1β was also purified as a HIS-tagged recombinant protein, and used to show it's reactivity with PDI. Finally, the first steps have been made in producing redox-state specific monoclonal antibodies. Some 61 were tested, with some warranting further research.

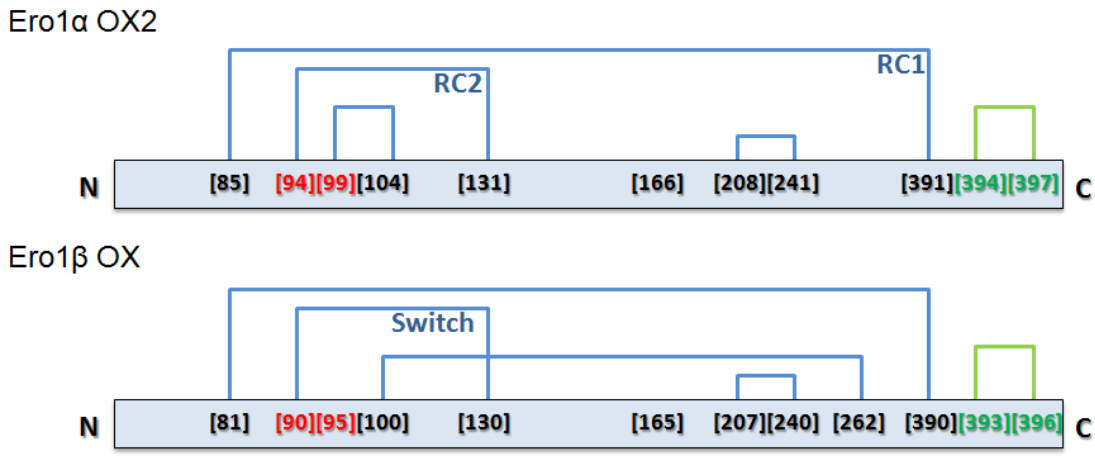

Figure 6.1 Stick diagram comparing the regulatory cysteines and N-glycan sites of Ero1α and Ero1β

Both Ero1α and Ero1β share similar arrangements of long-range disulphides. The numbers represent individual cysteines, paired in a disulphide bond using linear bridges. The red cysteines are the shuttle cysteines, the green cysteines are the active site. In the inactive OX2 state of Ero1α, the second pair of regulatory cysteines, RC2 eliminate the disulphide bond between Cys94-Cys99, rendering the protein inactive. In Ero1β, this disulphide switch is between Cys90-Cys130. (adapted from Appenzeller-Herzog *et al.*, 2008, Baker *et al.*, 2008 and Wang *et al.*, 2011)