



Durham E-Theses

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

WATSON, GRAEME

How to cite:

WATSON, GRAEME (2012) *EXPRESSION OF ENDOPLASMIC RETICULUM OXIDOREDUCTASES (EROS) AND THEIR ROLE IN THE GI TRACT*, Durham theses, Durham University. Available at Durham E-Theses Online: <http://etheses.dur.ac.uk/5945/>

Use policy

The full-text may be used and/or reproduced, and given to third parties in any format or medium, without prior permission or charge, for personal research or study, educational, or not-for-profit purposes provided that:

- a full bibliographic reference is made to the original source
- a [link](#) is made to the metadata record in Durham E-Theses
- the full-text is not changed in any way

The full-text must not be sold in any format or medium without the formal permission of the copyright holders.

Please consult the [full Durham E-Theses policy](#) for further details.

Academic Support Office, Durham University, University Office, Old Elvet, Durham DH1 3HP
e-mail: e-theses.admin@dur.ac.uk Tel: +44 0191 334 6107
<http://etheses.dur.ac.uk>

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

GRAEME RONALD WATSON

ABSTRACT

It has been shown that some ER redox enzymes are differentially expressed in stomach and oesophagus tissue. The tissues of the gastrointestinal system, which are subject to external changes of environment during the process of digestion, represent a novel area in which human ER oxidoreductases (Eros) can be studied.

Barrett's oesophagus is a common premalignant condition characterised by acid and bile reflux. We hypothesised that the development of metaplastic tissue in Barrett's may be associated with changes in the expression of Eros, and that the environment of gastric reflux could drive oxidative changes in the structure of Eros.

In this thesis, it is shown that Ero1 α is expressed at a higher level in OE33 oesophageal adenocarcinoma cells than in OE21 oesophageal squamous carcinoma cells. Ero1 β is not expressed in these cells. Altering pH or culture media or bile acid treatment does not cause any detectable changes in the expression or oxidation state of Ero1 α , Ero1 β or Protein Disulphide Isomerases (PDIs) in the OE21 and OE33 cell lines.

Human Ero1 β was produced as a recombinant HIS-tagged protein, which was inactive when thioredoxin was used as a substrate, but could oxidise PDI *in vitro*. Attempts were made to produce redox-state specific antibodies against either Ero1 α or Ero1 β . Ero1 α and Ero1 β -HIS recombinant proteins were used to produce hybridomas, which were tested for Ero1 α or Ero1 β specificity in rodent tissue and cell lines.

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

Graeme Ronald Watson

A thesis submitted at the University of Durham for the degree of
Doctor of Philosophy

School of Biological and Biomedical Sciences,
Durham University

September 2011

TABLE OF CONTENTS

1 INTRODUCTION	1
1.1 Overview	2
1.2 Protein synthesis and pre-ER events	3
1.3 Protein entry into the Endoplasmic Reticulum	6
1.3.1 Protein entry into the ER; the SRP and the translocon	6
1.3.1.1 Signal peptide cleavage	11
1.3.1.2 ER protein retention and retrieval	11
1.3.1.3 Glycoprotein folding, calnexin and calreticulin	12
1.4 Disulphide bond formation	19
1.4.1 Disulphide bond formation in Prokaryotes	22
1.4.2 Disulphide bond formation in Eukaryotes	25
1.4.2.1 Endoplasmic Reticulum Oxidoreductase	26
1.4.2.2 The yeast Ero, Ero1p	27
1.4.2.3 The Mammalian Eros; Ero1 α and Ero1 β	29
1.4.2.4 Protein Disulphide Isomerase, PDI	38
1.4.3 The Unfolded Protein Response and ERAD	46
1.5 Barrett's Oesophagus and Oesophageal Adenocarcinoma	48
1.5.1 Bile acids	50
1.5.2 Synthesis, structure and properties of bile acids	53
1.5.3 Studying gastric reflux using model systems	56
1.6 Hypoxia and ROS in gastric cancers	59
1.7 Aims of this thesis	62
2 MATERIALS AND METHODS	63
2.1 Antibodies	64
2.2 Molecular Biology	66
2.2.1 DNA Constructs used	66
2.2.1.1 Primers	66
2.2.2 Purification of plasmid DNA	67
2.2.3 Calculation of DNA concentration/yield	69
2.2.4 Reverse-Transcriptase PCR	69
2.2.5 Agarose gel electrophoresis	70
2.3 Cell Culture	70
2.3.1 Cellular Transfection	71
2.3.1.1 Transfection of oesophageal cell lines	72

2.3.2	Assessment of cell viability	73
2.3.2.1	Trypan blue assay	73
2.3.2.2	Crystal violet assay	73
2.3.3	Preparation of cell culture medium for treatment	73
2.3.4	Cell culture media pH range treatments	74
2.3.5	Cell lysis	74
2.3.6	Immunofluorescence	75
2.3.6.1	Seeding to coverslips	75
2.3.7	Sectioning of cell culture pellets	76
2.4	SDS-PAGE	76
2.5	Western Blotting	77
2.5.1	Screening monoclonal antibodies	78
2.5.1.1	Lysis of animal tissues	78
2.5.2	Determination of protein concentration	79
2.6	Protein gel staining	79
2.6.1	Protein Gel staining (Alternate Method)	80
2.6.2	Silver Staining	80
2.7	Protein Purification, Mutagenesis, and functional assays	81
2.7.1	Ero1L-B-HIS-GST Purification	81
2.7.2	PDI-HIS and TRX-HIS Purification	83
2.7.3	Centrifugal concentrating of protein solutions	84
2.7.4	Oxygen electrode recordings	85

3 CHARACTERISATION OF THE OESOPHAGEAL CELLS OE21 AND OE33, AND THEIR EXPRESSION OF ER OXIDOREDUCTASES 86

3.1	Introduction	87
3.2	Results	88
3.2.1	Characteristics and appearance of the cell lines HT1080, OE21 and OE33	88
3.2.2	Basal expression of oxidoreductases and PDIs in oesophageal cell lines	89
3.2.3	Transfection of HT1080, OE21 and OE33 cell lines with Ero1 α -myc	96
3.3	Using cell culture models of reflux and gastrointestinal cancer To explore expression of oxidoreductases and protein disulphide isomerases	99
3.3.1	Alteration of pH to determine the effect on cell viability and ER protein expression	99

3.3.2	Simulating reflux with the bile acids deoxycholic acid and chenodeoxycholic acid	108
3.4	Expression of PDI homolog, AGR2	116
3.5	Discussion	122
<u>4 EXPRESSION AND PURIFICATION OF ERO1B</u>		124
4.1	Introduction	125
4.2	Results	126
4.2.1	Ero1 β expression, detection of alternate splice forms	126
4.3	Protein purification	137
4.3.1	Purification of PDI and TRX	137
4.3.2	Purification of Ero1 β	142
4.3.2.1	Initial purification and functional tests of Ero1 β	142
4.3.2.2	Final purification and testing of Ero1 β	148
4.4	Discussion	157
<u>5 ANALYSING POTENTIAL ERO1 MONOCLONAL ANTIBODIES</u>		159
5.1	Introduction	160
5.2	Results	161
5.2.1	Supernatants	161
5.2.1.1	Validation of murine and rat tissues	163
5.2.1.2	Validation of Ero1 α and Ero1 β positive controls	166
5.2.1.3	Initial examples of hybridoma supernatants	169
5.2.1.4	Systematic testing of supernatants	173
5.2.1.5	Re-testing of potentially positive monoclonals	185
5.2.1.6	Re-testing 2.2C4 in cell immunofluorescence	191
5.3	Discussion	196

6 DISCUSSION	198
6.1 Differential Ero1 α expression between OE21 and OE33	199
6.2 Purification of Ero1 β	205
6.3 Ero1 β function	206
7 REFERENCES	210

LIST OF TABLES

Table 1.1: A summary of the 19 PDI family members (known to date)	45
Table 2.1 Antibodies used with dilutions for western blot	65
Table 2.2 Sequences of common primers	67
Table 4.1 Ero1 β alternate splice sequence primers	127
Table 5.1: Supernatants tested by ELISA and used in antibody screening	162

LIST OF FIGURES

Figure 1.1	Energy Landscapes in Protein Folding	4
Figure 1.2	Structure of an N- glycan	15
Figure 1.3	Disulphide Bond Formation	21
Figure 1.4	Stick diagrams representing Ero1 α and Ero1 β	31
Figure 1.5	Stick diagrams comparing Ero1 α and Ero1p structural disulphide components	32
Figure 1.6	Diagram showing proposed mechanism of disulphide formation in Ero1 α	36
Figure 1.7	Schematic representation of human PDI	44
Figure 1.8	Overview of bile acid synthesis	51
Figure 3.1	Appearance of the cell lines HT1080, OE21 and OE33 (normal light microscopy)	90
Figure 3.2	Schematic of non-reduced and non-reduced proteins in SDS-PAGE	92
Figure 3.3	Ero1 α can be reduced <i>in vitro</i> with the reducing agent dithiothreitol	93
Figure 3.4	Expression profiles of Ero1 α and PDI in HT1080, OE21 and OE33	94
Figure 3.5	Western blots from transfection experiments	98
Figure 3.6	HT1080, OE21 and OE33 pH 1 24 hour treatment	102
Figure 3.7	OE21 and OE33 pH 1 and 2 24 hour treatment compared to control	103
Figure 3.8	Trypan blue stained OE21 and OE33 pH 5-7 treated cells	104
Figure 3.9	Trypan blue stained OE21 and OE33 pH 5-7 treated cells	105
Figure 3.10	PDI expression in HT1080 cells following 24 hour pH 6.5-8.5 treatments	106
Figure 3.11	PDI and Ero1 α expression in OE21 and OE33 following pH 6.5-8.5 treatments	107
Figure 3.12	OE33 DCA treatments	110
Figure 3.13	OE33 DCA treatments	111
Figure 3.14	DCA time course treatments of OE33 and OE21 cells	112
Figure 3.15	Western blot for Ero1 α expression in CDC treated OE cells	114

Figure 3.16	PDI expression following CDC titration over 24 hours in OE21 and OE33	115
Figure 3.17	Alcian blue staining in HeLa, OE21 and OE33	118
Figure 3.18	Immunofluorescent staining of OE21 for PDI	119
Figure 3.19	Immunofluorescent staining of HeLa, OE21 and OE33 for PDI	120
Figure 3.20	Immunofluorescent staining of HeLa, OE21 and OE33 for AGR2	121
Figure 4.1:	Schematic showing the three putative splice variants of Ero1 β	127
Figure 4.2:	RT-PCR Validation of Ero1 β primers	128
Figure 4.3:	Expression of Ero1 β variants after tunicamycin treatment	130
Figure 4.4:	Expression of Ero1 β mRNA +/- tunicamycin and protein in oesophageal cell lines	131
Figure 4.5:	RT-PCR comparing expression of Ero1 β -16, 7 and 4 with BiP and actin following ER stress treatments	134
Figure 4.6:	RT-PCR comparing expression of Ero1 β -16, 7 and 4 with BiP and actin following bile acid treatments	135
Figure 4.7:	Purification of PDI in BL21 <i>E. coli</i>	139
Figure 4.8:	Final purification of PDI in BL21 cells	140
Figure 4.9:	Purification of thioredoxin	141
Figure 4.10:	Predicted running sizes of Ero1 β recombinant proteins	143
Figure 4.11:	Initial purification steps in the production of Ero1 β -HIS	144
Figure 4.12:	Coomassie gel showing Ero1 β -HIS-GST stability	146
Figure 4.13:	Thrombin cleavage stability test at 4 °C	147
Figure 4.14:	Outline of the optimised Ero1 β purification protocol	151
Figure 4.15:	Optimised purification of Ero1 β -HIS	152
Figure 4.16:	Final purification of Ero1 β , showing GST removal with Thrombin	153
Figure 4.17:	Comparison of Ero1 β -HIS generated by the two protocols	154
Figure 4.18:	Assay of TRX and Ero1 β -HIS	155
Figure 4.19:	Oxygen electrode recording of the reaction between Ero1 β -HIS and reduced PDI in the presence of GSH	156
Figure 5.1:	Coomassie staining of cell and tissue lysates	164
Figure 5.2:	No cross-reactivity of the anti-mouse secondary antibody with mouse tissues	167

Figure 5.3:	Verification of Ero1 α and Ero1 β positive controls	168
Figure 5.4:	Initial test with antibody, 3.3C4 (Ero1 β –HIS-GST)	171
Figure 5.5:	Initial test with antibody 4.5B5 (Ero1 β -HIS)	172
Figure 5.6:	Series 1 (Ero1 α wildtype) supernatants tested in Western blot	174
Figure 5.7:	Series 2 (Ero1 α C397A) supernatants tested in Western blot	176
Figure 5.8:	Series 2 (Ero1 α C397A) supernatants tested in Western blot	177
Figure 5.9:	Series 3 (Ero1 β -HIS-GST) supernatants tested in Western blot	178
Figure 5.10:	Series 3 (Ero1 β -HIS-GST) supernatants tested in Western blot	179
Figure 5.11:	Series 4 (Ero1 β -HIS) supernatants tested in Western blot	181
Figure 5.12:	Series 4 (Ero1 β -HIS) supernatants tested in Western blot	182
Figure 5.13:	Series 4 (Ero1 β -HIS) supernatants tested in Western blot	183
Figure 5.14:	Series 4 (Ero1 β -HIS) supernatants tested in Western blot	184
Figure 5.15:	Retest of Series 2 (Ero1 α C397A)	187
Figure 5.16:	Re-test of Series 3 (Ero1 β -HIS-GST)	188
Figure 5.17:	Re-test of Series 4 (Ero1 β -HIS)	189
Figure 5.18:	Re-test of Series 4 (Ero1 β -HIS)	190
Figure 5.19:	Immunofluorescence signal of ER tracker in HeLa and OE21	193
Figure 5.20:	Immunofluorescence signal of PDI in HeLa and OE21	194
Figure 5.21:	Immunofluorescence signal of PDI in HeLa and OE21	195
Figure 6.1	Stick diagram comparing the regulatory cysteines and N-glycan sites of Ero1 α and Ero1 β	209

LIST OF ABBREVIATIONS

ADP	adenosine diphosphate
AGR2	anterior grade homolog 2
AMS	4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid
APP	amyloid precursor protein
APS	ammonium persulfate
AS	active site
ATF6	activating transcription factor
ATP	adenosine triphosphate
BiP	binding protein, aka Grp78
bp	base pairs
BSA	bovine serum albumin
BSEP	bile salt export pump
bZIP	basic leucine zipper domain
CA	cholic acid
cAMP	cyclic adenosine monophosphate
CD	cell adhesion molecule: cluster of differentiation
CDCA	chenodeoxycholic acid
cDNA	complementary DNA
CDX2	caudal-type homeobox 2
CFTR	cystic fibrosis transmembrane conductance regulator
CLAP	chymostatin, leupeptin, antipain and pepstatin
CMC	critical micelle concentration
CoA	coenzyme A
CPY	carboxypeptidase Y
CRE	cAMP-response element
CYP	cytochrome P
DAPI	4',6-diamidino-2-phenylindole
DCA	deoxycholic acid
DMEM	Dulbecco's modified Eagles's medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDEM	ER degradation enhancing 1,2-mannosidase like protein
EDTA	ethylenediaminetetraacetic acid
EGF	endothelial Growth Factor
Endo H	endoglycosidase H
ER	endoplasmic reticulum

ERAD	ER-associated degradation
ERD2	ER lumen protein retaining receptor
Ero1 α	endoplasmic reticulum oxidoreductase α
Ero1 β	endoplasmic reticulum oxidoreductase β
ERp	endoplasmic Reticulum Protein
ERSE	ER stress response element
FAD	flavin adenine dinucleotide
FCS	fetal calf serum
GDEA	gastroduodenooesophageal anastomosis
GDP	guanosine 5'-diphosphoglucose
GI	gastrointestinal
Gls I	glucosidase I
Gls II	glucosidase II
GORD	gastro-oesophageal reflux disease
GPX	glutathione peroxidase
Grp78	glucose regulating protein, aka BiP
GSH	reduced glutathione
GSSG	oxidised glutathione
GST	glutathione S-transferase
GT	UDP-glucose:glycoprotein glycosyltransferase
GTP	Guanosine-5'-triphosphate
H ₂ O ₂	hydrogen peroxide
HA	influenza virus hemagglutinin
HIF-1	hypoxia-inducible factor 1
HIS	Histidine tag
HSP	Heat Shock Protein
Ig	immunoglobulin
IP	immunoprecipitation
IP3R1	inositol triphosphate receptor type 1
IPTG	isopropyl β -D-1-thiogalactopyranoside
IRE1	inositol requiring kinase 1
LB	lysogeny broth
LCA	lithocholic Acid
MAM	mitochondrial membrane associated ER membrane
MAPK	mitogen-activated protein kinase
MEM	minimum essential medium
MES	2-(N-morpholino)ethanesulphonic acid
MHC	major Histocompatibility Complex
Mns I	mannosidase I

MNT	MES-NaCl-Tris lysis buffer
mRNA	messenger ribonucleic acid
NEF	nucleotide exchange factor
NEM	N-ethylmaleimide
NF- κ B	nuclear factor kappa-B
NI-NTA	nickel-nitriloacetic acid
NMR	nuclear magnetic resonance
OD	optical density
ORF	open reading frame
OST	oligosaccharyl transferase
PAGE	polyacrylamide gel electrophoresis
PBD	protein binding domain
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDI	protein disulfide isomerase
PERK	protein kinase RNA-like endoplasmic reticulum kinase
Prx	peroxiredoxin
pK _a	acid dissociation constant
QSOX	quiescin-sulphydryl oxidase
RAMP	receptor activity-modifying protein
RC1	regulatory cysteines 1
RC2	regulatory cysteines 2
RER	rough endoplasmic reticulum
RNA	ribonucleic acid
ROS	reactive oxygen species
Rpl Proteins	ribosomal proteins
RPMI	Roswell Park Memorial Institute medium
rRNase	reduced ribonuclease
RT-PCR	reverse transcriptase PCR
SC	shuttle cysteines
SDS	sodium dodecyl sulphate
SER	smooth endoplasmic reticulum
SOD2	superoxide dismutase 2
SRP	signal recognition particle
TAE	tris base, acetic acid and EDTA
TAP	transporter associated with antigen processing
TBS	tris-buffered saline
TCA	trichloroacetic acid
TEMED	N,N,N',N'-Tetramethylethylenediamine

TM	transmembrane domain
TRAM	translocating chain-associated membrane protein
TRX	thyoredoxin
UPR	unfolded protein response
VEGF	vascular endothelial growth factor
XBP1	x-box-binding protein-1

DECLARATION

I declare that the experiments described in this thesis were carried out by me in the School of Biological and Biomedical Sciences, University of Durham, under the supervision of Dr. Adam M. Benham and Mr YKS Viswanath. This thesis has been composed by myself and is a record of work that has not been submitted previously for a higher degree.

This copy has been supplied for the purpose of research or private study on the understanding that it is copyright material and that no quotation from the thesis may be published without proper acknowledgement.

Graeme Ronald Watson

ACKNOWLEDGEMENTS

I would like to take this opportunity to thank a number of people who have helped me throughout my period of study. First, I must thank my supervisors Dr. Adam Benham and Mr YKS Viswanath for providing me the opportunity to do this PhD at The University of Durham, in association with the upper GI surgical team of James Cook University Hospital, Middlesbrough and Dr Anjan Dhar of the upper GI Endoscopy Unit at Bishop Auckland General Hospital. I would also like to thank them for their support, help and guidance throughout this time.

I would also like to thank Dr Nick Hole for co-supervision and his support and guidance during Dr Adam Benham's research sabbatical in Osaka, Japan during 2009. Thanks too must also go to Dr Andrew Lemin and Dr Marcel van Lith for day-to-day help and support in the lab.

I would also like to thank the lab group of Professor Neil Bulleid, including Dr Karl Baker, Dr Cathy Jessop, Dr Joe Chambers, Ms Rae Jackson, Dr Tim Tavender, Dr Shweta Tiwari for support and guidance during my time there, the basis of much of the data in Chapter 4. In addition, further thanks to Swee Kim Ang, Romina Durigon, Dr Bruce Morgan, Jennifer Springate, Dr Lisa Swanton and Dr Lydia Wunderley of the University of Manchester for making me feel welcome in a new city.

Further thanks to for the staff of Durham University's Department of Biological and Biomedical Sciences, who have proved to be good friends and sources of support including Ms Val Affleck, Dr Catherine Bruce, Dr Susanne Cormack, Mrs Leslie Edwards, Mrs Jillian Lynn, Ms. Bridie Murray, Dr Mags Pullen, Dr Susan Pyner, Mrs Ann Tobin and many more over the years.

DEDICATION

For my parents, Ron and Margery Watson for their ongoing love and support, without whom I would not be the man I am today.