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Stress and Redox-dependent Chaperone
Interactions in the Endoplasmic Reticulum
in Barrett's Oesophagus

A thesis submitted for the degree of Master of
Science (Research)

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Submitted: 2016

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Acknowledgments

I would like to thank Dr Adam Barber and Helen Lake for helping me going and all the support they have granted me. If it wasn't for them this thesis wouldn't have been completed.

I would also like to thank Dr Yvonne Kelly for her co-supervisory advice and James Cook University Hospital for their support and collaboration with this project.

I would like to thank the Barrett's support group for helping me feel the friends and family. I would like to thank Nicola, Michelle, Ben and Sarah for their advice and support throughout.

Abstract

Genetic Oesophageal (EO) is an acquired condition which alters the normal structure of the distal oesophagus. EO is reported as the only histoprecursor lesion for Oesophageal Adenocarcinoma (EAC) with an annual conversion rate of approximately 3-5%. There is a strong correlation between acid reflux and the development of EO, with patients with EO having a high incidence of bile acids in their refluxate. Bile acids have been implicated in EAC onset and disruption of protein folding which may be important in the transition from EO to EAC.

Oesophageal GP72 has been shown to protect oesophageal cells from bile acid induced stress. However, there is evidence to suggest that GP72 is down regulated in hyperproliferation of the premalignant EO. Plectin also has an association with EO, has not been investigated fully in EO and EAC. Finally AGS2 is a member of the FGD family which has been implicated in a number of cancers and may play a role in the development of EAC.

This study did not find any reliable expression of GP72 in EO cells or in tissue samples of EO and EAC. Western blotting of EO cells showed a difference in relative protein between EO19 and EO33 cells and HC showed expression of Plectin in gastric-like cells in EO but none in EAC tumour cells. AGS2 did not appear to be upregulated in the normal oesophageal but was present in specific the malignant cells in EO which appeared to correlate closely associated with tumour cells in EAC. Cellular experiments showed that AGS2 forms a dose dependent interaction with EO cells. Taken together this may suggest a relationship between Plectin and AGS2 and the transition of EO to EAC.

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1.5 The Structure and Histology of the Oesophagus

The oesophagus is a muscular tube which begins at the pharynx and ends at entrance to the stomach comprising, from the outside in, four muscle layers, longitudinal and circular, a sub-mucosal layer, the muscularis mucosa layer, the lamina propria and finally the epithelial layer. The epithelium of the oesophagus consists of a stratified layer of squamous epithelial cells, containing keratinized filaments linked to tubular mucous, submucosal glands and changes to a non-keratinized columnar epithelium at the gastro-oesophageal junction (Masthuur, 2004).

Viewed histologically, the epithelial cells, at the luminal edge of the oesophageal epithelium, appear flattened, becoming gradually larger as the cells transition towards the basal axis. Many of these cells are rich in glycogen. The basal part of the oesophageal epithelium comprises of a variable number of layers of rectangular or cuboidal cells which lack glycogen and is scattered with melanocytes and melanin-laden cells. Below the oesophageal epithelium is the lamina propria. The junction between the basal epithelial layer and the lamina propria is irregular and irregular of the lamina propria extending towards the lumen lumen. The lamina propria consists of a glycosaminoglycan matrix in which fibroblasts and loosely arranged collagen fibres are embedded, along with lymphocytes, macrophages and occasionally plasma cells and mast cells. The muscularis mucosa, below the lamina propria, is variable in thickness, being thicker towards at the gastro-oesophageal junction. The muscle fibres of the muscularis mucosa, appear to be arranged regularly in the upper portion of the oesophagus transitioning to continuous sheets of longitudinal and circular smooth muscle in the lower part (Gowans & Lane, 1987).

1.2 Barrett's Oesophagus

Barrett's Oesophagus (BO) is an acquired condition which alters the normal structure of the distal oesophagus. In BO the stratified squamous epithelium of the oesophagus, near the oesophago-gastric junction, is replaced by a metaplastic columnar epithelium which can comprise three different morphologies. These are: simple gastric fundic type epithelium containing goblet and chief cells; transitional epithelium with cardiac-mucosal secretory glands and specialised columnar epithelium with intestinal like goblet cells. However, there is some disagreement in clinical practice as to what constitutes BO. American gastroenterologists require the presence of intestinal columnar epithelium for a positive diagnosis of BO, whereas British guidelines consider the possibility of diagnosing BO on the presence of gastric metaplasia only (Peters, et al., 2014).

BO is itself a relatively benign and could be considered as an adaptive mutation in response to refluxing gastric contents, containing acid and bile. Chirbaev et al., citing a genetic and proteomic approach discovered that there were over 2000 genes differentially expressed between 22 paired samples of Oesophageal squamous epithelium and BO samples including liver shock proteins and those that inhibited cell death, such as BCL2 and BCL2L1 and those such as TP53. Chirbaev, et al., 2012. Clinically, BO is reported to be the only known precursor lesion for Oesophageal Adenocarcinoma (OAC) (Peters, et al., 2014) (Hudson & Gilbey, 2014).

Oesophageal cancer is the 7th most common cancer in men, the 4th most common in women (Peters, et al., 2014) and has shown an exponential increase in overall incidence over the last three decades (Hudson & Gilbey, 2014). Oesophageal

cancer in aggression in males and in metastasis and lymphatic invasion in early stages which results in a low response resulting in Oesophageal cancer being the 4th and 7th highest cause of cancer related deaths in men and women respectively with a 5 year survival rate of 5-10% (B. Paton, et al. 2014) (Mason & Colburn, 2014). Studies of ESO have a 2.5% annual incidence rate in CRC however this has been an increase in the incidence of ESO and a decrease in age of presentation. According to the United Kingdom Cancer Registry (Haines, 2014) 27% of the 12,000 registered ESO patients were diagnosed before the age of 55 (Haines & Coleman, 2014).

The development of ESO via CAC is thought to be a multi-step process where the metaplastic ESO epithelium is thought to sequentially transition through a low grade dysplastic epithelium to a high grade dysplastic epithelium and finally into invasive CAC (Ch, et al. 2010). The underlying mechanisms which allow the transition are unclear. One possible theory proposed is transdifferentiation, a change of one cell type into another. The early developing Oesophagus is composed of simple columnar cells which differentiate into the normal squamous epithelium during development. As the oesophagus develops the appearance of squamous epithelial cells coincides with the down regulation of its signaling, which provides the information required by endocytic cells for proper differentiation. Wang Q et al proposed that Oesophageal injury from acid and bile reflux could cause aberrant activation of the Wnt signaling pathway and this could be the cause of the transdifferentiation to columnar cells in ESO. Wang et al showed that over expression of Sonic Hedgehog in mouse Oesophageal epithelium induces columnar gene expression, in addition Wang et al found 70 cases of ESO with increased Wnt signaling and no progression that occurred in activation of the Wnt signaling pathway.

could be the cause of the transdifferentiation to columnar cells in BC (Wang, et al., 2010).

While transdifferentiation explains the observed replacement of the normal squamous epithelium with columnar epithelium, which is seen in BC, it fails to explain the rise in other cell types seen in BC. Given that the cell types observed in BC resemble those represented in residual tissue it is possible that undifferentiated stem cells undergo transdifferentiation into an intestinal type stem cell. The exact location of the stem cell which give rise to BC is unknown although a number of candidates exist. Certainly squamous metaplasia stem cells in the basal epithelial layer, stem cells present in the submucosal glands (Shen & Tsai, 2012) and finally gastric stem cells which have originated from the gastric cardia at the gastro-oesophageal junction (Quain, et al., 2012).

There is also evidence to suggest other possibilities. Wang, et al showed that a diverse population of embryonic epithelial cells persist in adult mice and human at the approx. oesophageal junction and can respond to the rise of squamous epithelium (Wang and papaleo (Wang, et al., 2011). Additionally, a rat model of Barrett's Oesophagitis (BO) showed evidence that multipotential stem cells originated from bone marrow via the blood to the site of the reflux-damaged epithelium (Saito, et al., 2008).

While environmental and lifestyle factors, appear to be a major contributive factor in the development of BO (Table 1.1) there appears to be some evidence of a possible genetic relationship in the development of BO and OAC. Studies have shown that within ethnic populations of BC and OAC, approximately 7% of families share some familial clustering (Peters, et al., 2014).

Table 1.1 Risk factors for the development of Ovarian Cystadenomas (OVC) (Peters, et al., 2016; Samuels & Beach, 2010; Samuels & Beach, 2010)

Risk factor	Biological Mechanism
Obesity (BMI > 30)	Obesity is associated with increased levels of circulating insulin and insulin-like growth factor (IGF) and is associated with increased levels of estradiol.
Family history of OVC	Family history of OVC is associated with increased levels of circulating estradiol and is associated with increased levels of estradiol.
Endometriosis	Endometriosis is associated with increased levels of circulating estradiol and is associated with increased levels of estradiol.
Smoking status	Smoking is associated with decreased levels of circulating estradiol and is associated with decreased levels of estradiol.
Menstrual cycle length	Menstrual cycle length is associated with increased levels of circulating estradiol and is associated with increased levels of estradiol.
Diets	Diets high in fat and low in fiber are associated with increased levels of circulating estradiol and is associated with increased levels of estradiol.
Reproductive history	Reproductive history is associated with increased levels of circulating estradiol and is associated with increased levels of estradiol.
Progeny Characteristics	Progeny characteristics are associated with increased levels of circulating estradiol and is associated with increased levels of estradiol.

A number of genome-wide association studies have identified loci that correlate with increased disease risk, the most notable of which are described below.

The Esophageal Adenocarcinoma Genetics Consortium tested over 521,000 single nucleotide polymorphisms (SNPs) and discovered an association with a SNP on chromosome 5q21 within the major histocompatibility complex, called HLA-DQA1. This association was replicated in a SNP on chromosome 15q21.08 (SNP) at 1.14, which codes for FOXP1. A protein thought to be associated with esophageal development (The Esophageal Adenocarcinoma Genetics Consortium and the Wellcome Trust Case Control Consortium, 2010). Another group headed by David Levine found three genetic SNPs, 18p11.23 (CTCF) associated with oncogenic activity when aberrantly activated, 5q22.3 (E2F1) a transcription factor related to esophageal specification and FOXP1 on 5q14 is a regulator of esophageal development (Levine, et al., 2015). Whatever the driver of the change in esophageal cancer is, it appears to be evidence of a progressive increase in methylation and a subsequent accumulation of general genomic instability and oncogenic events, such as the loss of the tumour suppressor genes CDKN2A and TP53 (Rosenblatt, et al., 2015) (Shahar, et al., 2015).

Although there is evidence of a genetic cause of people who are at an increased risk of the formation of esophageal cancer, one of the main risk factors for esophageal cancer is the presence of acid reflux (Table 1). Gastroesophageal Reflux Disease (GERD) and esophageal adenocarcinoma are strongly correlated with the development of esophageal cancer. In fact, more than 10% of those presenting with GERD and 20% of those with esophageal adenocarcinoma. Additionally they demonstrated that the high pH of

segments correlated with the exposure to acid in the chemotherapy. They found that patients with higher segments of BO experienced significantly less exposure to reflux acid than those with larger segments of BO both when upright or supine, concluding that duration of acid exposure is an important factor in determining the weight of the Barrett's mucosa. (Faria, et al., 2007). Their approach has a strength in that it considered both Barrett's-resected acid reflux and BO and patients with BO have a high incidence of bile acids in their refluxes (Fahure, et al., 2011) implicating bile acids as etiologic agents in cancers of the gastrointestinal tract (Berman, et al., 2006). Choudhry et al demonstrated that a bile acid cocktail comprising of the equimolar salts of glycocholic acid, taurocholic acid, glycodeoxycholic acid, glycochenodeoxycholic acid and deoxycholic acid acidified to pH 1 could reduce oxidative stress in Human Barrett's Esophagus tissue. In addition they showed that the combination of bile acids and aspirin caused an increase in oxidative stress and oxidative DNA damage, shown by an increase in 8-hydroxy-deoxyguanosine, a marker for oxidative DNA damage in BO, CagA+ and HET-1A cells (Dharwadkar, et al., 2007). The bile acid deoxycholic acid has also been implicated as a carcinogen in experimental cancer by indirectly damaging DNA through the production of reactive oxygen and reactive nitrogen species (ROS/RNS) (Berman, et al., 2006) and with oxidative and Endoplasmic Reticulum (ER) associated stress, protein misfolding and calcium (Ca²⁺) imbalance in human esophageal bar disease (Berman, et al., 1995). These associations suggest that acid reflux and bile reflux on the ER may be important in the transition from BO to DAC.

1.3 The Endoplasmic Reticulum

The endoplasmic reticulum (ER) is a continuous membrane system, interconnected with the nuclear envelope (Figure 1-1). It is composed of a single membrane sheet, forming a single continuous surface which has various domains that perform different functions, including protein synthesis, folding, transport to the Golgi and lipid synthesis (Schwarz & Bosser, 2016). The ER is continuous with the nuclear envelope (NE) and consists of two main domains, the rough endoplasmic reticulum (RER) and the smooth endoplasmic reticulum (SER). The NE surrounds the nucleus, it consists of an inner nuclear membrane (INM) and an outer nuclear membrane (ONM). These are large integral membrane proteins by the inner nuclear membrane space (Sjoglin & Vinkler, 2015). The NE acts as a barrier to selectively control the entry and exit of molecules to the nucleus. Transport of molecules between the nuclear and cytoplasmic compartments is facilitated by nuclear pore complexes that bridge the INM and ONM (Schwarz & Bosser, 2016). The ER branches out from the ONM as an interconnected network of cisternae comprising the RER and the SER (Figure 1-1) with the conformationally stacked, flat cisternae of the RER located nearest the NE (Schwarz & Bosser, 2016) (Sjoglin & Vinkler, 2015). The RER comprises of flat lamellae with an intervening space which varies in size. The lamellar space is considerably around 50 nm in mammals and 30 nm in yeast (Schwarz & Bosser, 2016) and the cisternae of the RER are connected by tubular membrane sheets (Theriot et al., 2012). The RER is defined by the high density of ribosomes on its cytoplasmic surface and its function is the synthesis, folding and modification of proteins destined to be secreted or membrane bound (Schwarz & Bosser, 2016).

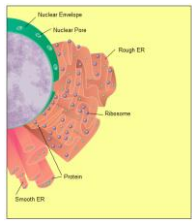


Figure 2.1 The Endoplasmic Reticulum. A graphical representation of the ER. The left side shows the rough endoplasmic reticulum (studded with ribosomes) and the right side shows the smooth endoplasmic reticulum. Adapted from (Shelton 2016)

The SR consists of highly conserved and smooth network of interconnected tubules and is defined by the liver density of membranes (Fagan & Yaffe, 2013). The SR is highly dynamic and constantly rearranging and is considered to be the primary site of lipidogenesis (Schwartz & Blobel, 2016).

1.4 Protein entry into the Endoplasmic Reticulum

A newly synthesized, nascent protein chain exists in a conformation determined by its amino acid sequence. Many nascent proteins, in order to assume a form that is biologically active, must undergo some form of protein folding and this process must be reproducible in order that every protein translated from a particular mRNA sequence results in a form fit for purpose.

Proteins that are expressed at the cell surface, are part of the secretory pathway or are destined to be inserted into organelles in the ER, have a signal sequence which is located at the N-terminus of the emerging nascent polypeptide chain (Figure 1.2). The signal sequence is usually around 25 amino acids in length and comprises of 12 hydrophobic amino acids preferentially formed in an alpha-helix the exact composition of which is variable in length, charge and amino acid composition (Dingler, 1985).

The signal sequence binds to a Signal Recognition Particle (SRP), which facilitates the targeting of the translating protein to the ER membrane (Figure 1.2A) (Pawl, 2005). The SRP is a heterodimeric complex consisting of 7 alpha-helices, 4 beta-strands and approximately 300 nucleotides, formed into a stable ordered secondary structure in complex with six protein subunits (SRP α , 14, 16, 54, 68 and 72) and can be thought of as consisting of two structural domains, named the A- and B-domains (Holtz, 1985). The A-domain is made up from helices 3-5 of the SRP, and SRP α and 6 subunits.

The G domain comprises of the rest of the 725, 686 and the 597-611, in 2005, the 597-611 and the 612-625 residues. Of all the subunits 597-611, the function of the SPP, in that it is involved in interaction with the ribosome, signal sequence recognition and interaction between the SPP and the ER membrane SPP receptor (SR) (Hogari, et al., 2010). Structurally SPP is composed of 3 main domains: an N-terminal, the hydrophobic (H) domain at the C-terminal, a CTPase domain (C) and a N-terminal helical domain (N). The M domain binds the nascent chain signal sequence and the N and G domains are involved with the interaction with the SR. The M and H domains are joined by an highly conserved and flexible linker which permits the subunit to undergo structural rearrangement and allow communication between the domains (Hogari, et al., 2010) (Hogari, et al., 2010).

Unique to the subunit SPP, binding of the signal sequence to the SPP is a subset of the SPP's full domain, in addition of being a receptor event in the recognition of the nascent chain. Experiments with SPP mutants compromised in their ability to arrest elongation have demonstrated defects in translocation, suggesting that the arrest capability of the SPP depends on a subset from the nascent chain domains complex with the RNC bound to the translocator (Hogari, et al., 2010).

The SPP binds to the Ribosome Nascent Chain Complex (RNC) with low affinity. However, it is only the presence of an exposed signal sequence that leads to high affinity binding through the M domain of the SPP subunit of the SPP (Hogari, et al., 2010). Binding of the signal sequence causes a conformational change in SPPs which is thought to promote receptor engagement (Hogari, et al., 2010).

Once formed the DMC-SRP complex is targeted to the ER membrane bound SR. The SR (Figure 1.24) complex of two subunits, namely SRα and SRβ. SRβ is a 30kDa integral membrane protein anchored to the ER membrane via a single transmembrane domain. SRα is a 70 kDa protein that is tightly bound to SRβ and its transmembrane domain. Its C-terminal region includes a CTPase domain and has a high homology with the NC domain of SRP54 (Nguyen, et al., 2013). SRα also has a CTPase domain that only interacts with SRβ. SRα domain when it is in a CTP bound state. Schwartz and Blobel suggest that the CTP cycle of SRβ may control the association and dissociation of the SR (Schwartz & Blobel, 2005). The SRα sub-unit of the SR forms the interaction between the SR and the SRP. The NC domain of SRP54 forms a pseudo homodimer with the SRα domain only when the CTPase domains of both SRP54 and SRα have bound CTP (Nguyen, et al., 2013). There is also some evidence to suggest that location of the formation of the SRP-CR complex provides some proof reading ability. In that only the binding of genuine CR targeting sequences to the SRP promote rapid and stable SRP-CR dissociation (Zhang, et al., 2016).

Protein entry into the ER is facilitated by the SecY1 translocation complex (Figure 1.25). The SecY1 complex is a heterotrimer of two highly conserved subunits α and γ and a less well conserved β subunit. The α subunit, which forms the pore that accommodates the translocating nascent chain during translocation across the ER membrane (Fig. 1.25). SRα, has an immunoglobulin (Ig) domain and can be thought of as being in two distinct states, TM 1-6 and TM 7-10. The TM 6 are arranged to form a central pore, with a cytosolic loop between TM 5 and 6, which functions as a bridge allowing the opening of the pore. The luminal side is occluded by a plug

formed by a sub-domain of the 2nd transmembrane domain. The β subunit is formed by ER chaperone disulfide isomerase across the back of the α subunit and has a role in opening the luminal side of the pore complex to allow protein entry in the ER lumen. The β subunit likely corrects the α subunit and its exact function has not been fully elucidated (Plym-Forsell et al., 2010).

Shipping of the PDC to the Golgi/TransGolgi (Figure 1.12) involves opening of the pore complex and passage of the nascent protein chain into the ER lumen. Evidence suggests that the nascent chain signal sequence binds at the cytosolic side of the translocator and forms a hairpin-loop with the nascent protein as released from the PDC. The signal sequence can then be released to signal peptide isomerase (Spatz) (Figure 1.12) (Shew et al., 1988). However there is also evidence that suggests that initially the signal sequence may be inserted back first into the pore, in the case at least until the signal sequence flips inside the pore, into a hairpin-loop leading to the translocation of the cytosolic residues of the nascent chain and may require the introduction of accessory proteins SecE2B and ERJ (Plym-Forsell et al., 2010) (Chen et al., 2011). As translocation moves to completion, the nascent protein chain is released by the ER chaperone gp78 (Bai) (Figure 1.12) and pore co-receptors are modified, folded and stabilized by the addition of intermolecular disulfide bonds between chains of a single protein or intermolecular disulfide bonds between separate chains.

1.5 Oxidative Protein Folding in the ER

Disulfide bond formation is necessary to confer stability for secreted proteins, when they are exposed to the extracellular environment or, in the case of organelles

disulfide proteins, although specific endoplasmic compartments (Baldwin, 2012). The formation of disulfide bridges as a chemical process, chain is transferred to the ER. The reduction of a nascent chain into its secondary structure brings cysteine residues close enough together for the formation of disulfide bonds. The extent of the ER is more oxidizing environment than the cytosol and as such favors the oxidation of free thiol groups (SH) and the formation of disulfide bonds (S-S). However, correct disulfide bond formation requires enzymatic help to promote bond formation between cysteine residues and to remove those disulfide bonds that may form during folding that are not required by the proteins that form (Baldwin, 2012) (Bioscience & Biotech, 2011). Control to the cycle of oxidation and reduction in the protein disulfide isomerase (PDI) family of enzymes (Baldwin, 2012).

The PDI family of enzymes have the ability to serve as molecular chaperones and to act as oxidoreductases and as isomerases, forming, breaking, and rearranging disulfide bonds within their protein clients (Figure 1.34) (Bertman, 2012).

Formation of disulfide bonds requires that PDI family members be oxidized. There are a number of specific oxidases that, when being oxidized, can introduce disulfide bonds themselves into client proteins, not specifically within PDI family members (Baldwin, 2012), with the exception of quiescetin sulphydryl oxidase (QSOX), a Golgi localized protein, which has been shown to be able to introduce disulfide bonds without the aid of additional partners. However, being localized to the Golgi apparatus it's thought not to be involved in soluble protein folding (Chen, Wang, et al., 2007).

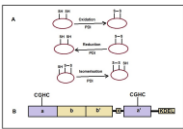


Figure 1 Schematic representation of PPA activation on linear polymer chain in water. PPA stands for 1,3-bis(3-phenylpropyl) carbodiimide, whereas the number of hydroxyl groups. (A) PPA activation on terminal hydroxyl, (B) activation of PPA on terminal hydroxyl, (C) and (D) activation of PPA on terminal hydroxyl, (E) PPA activation on terminal hydroxyl, (F) and (G) activation of PPA on terminal hydroxyl.

Structurally, the amino-terminus of PDI is the thioester domain. PDI contains two active thioester domains, a and a' as well as two disulfide B and B' (Figure 1.16). The a' and a' domains are separated by an α -helix region which contains a hydrophobic cap that can regulate ligand binding and transamidation (Bertram, 2012). The active site of PDI family member usually contains a Cu²⁺ motif, which is normally CXXC in PDI (Bertram, 2012). The intermolecular disulfide bond in the Cu²⁺ motif has a high reduction potential compared to that of unbound client proteins allowing the ready donation of its disulfide bond to a protein with a reduced redox potential (2012).

The mechanism of substrate protein folding can be considered as a series of oxidation reactions between PDI family members and different substrates. The main oxidase is ER Oxidoreductase 1 (ERO1) but both Oxidoreductase 2B (ERX2B) and Peroxisomal H₂Oxidase have also been suggested as capable of oxidizing PDI (Figure 1.4) (Giles, 2012). ERO1 and PDI have the main pathway for disulfide bond formation in the ER. ERO1 is a glycosylated tetraspanner associated with the luminal side of the ER membrane. It has two homologues, in *Saccharomyces cerevisiae* (ERO1) and *Drosophila melanogaster* (ERO1). These homologues appear to undertake similar functions but have different tissue distribution (Poggen, et al., 2002). ERO1 has two catalytic sites each housing a pair of cysteines. The 'outer' site is located close to the bound FAD cofactor and the 'inner' site is located on a flexible loop. ERO1 promotes disulfide bond formation by utilizing the oxidation potential of molecular oxygen and its bound FAD cofactor to generate intermolecular disulfide bonds that it can transfer to the client protein via PDI. This is achieved by a series of thio-disulfide bond exchange reactions (Figure 1.16). Complexed by ERO1 enables the direct oxidation of PDI by the thioester site available to

addition of the reduced state, via its normal translocation exchange function, the whole and the active sites. Molecular oxygen and PDI in turn re-oxidize the active site producing H₂O. (Bullard, 2012) (Saito & Kawan, 2008).

ERD1 is not the only ER resident protein that is capable of oxidase activity and has been found to interact with PDI. Both Nguyen et al and Wang et al have demonstrated that GP17 and GP46 have the ability, in the presence of PDI, to oxidize PDI and facilitate disulfide bond formation in their position (Nguyen, et al, 2011) (Wang, et al, 2014). GP7 and 8 are members of the GP4 family, GP7 is a soluble ER protein and GP8 is a type 1 membrane protein (Bullard, 2012). Nguyen et al demonstrated evidence that both GP7 and GP8, upon ER localized glutathione peroxidase cleavage associated with ERD1 and neither demonstrated substantial glutathione peroxidase activity, instead they demonstrate PDI peroxidase activity. In they show a preference for accepting electrons from PDI. In vivo studies corroborated the addition of GP7/8. PDI not previously to a reduced oxidase protein resulted in disulfide formation and rapid reducing rate in native state. They also demonstrated that addition of GP7/8 to a mix of PDI, ERD1a and reduced glutathione (GR) resulted in a significant increase in disulfide formation. Together this information provides evidence to suggest that GP7 and GP8 form part of a pathway designed to efficiently utilize the peroxide produced by the main ERD1-PDI pathway for disulfide bond formation (Nguyen, et al, 2011).

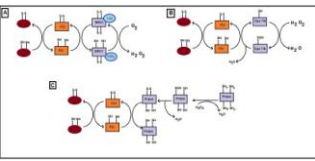


Figure 1.1 Mechanism of the interaction between the endonuclease SPVNS and PACE. (a) The protein SPVNS (red) binds to the DNA double helix (grey) and PACE (orange) binds to the DNA. (b) The protein SPVNS (red) binds to the DNA double helix (grey) and PACE (orange) binds to the DNA. (c) The protein SPVNS (red) binds to the DNA double helix (grey) and PACE (orange) binds to the DNA.

This evidence is further supported by Wang et al., who showed that GP72 could allow the peroxide generated by PDI1 to oxidize disulfide isomerase protein both *in vitro* and *in vivo*. The mechanism by which, they suggest, GP72 allows H_2O_2 to oxidize PDI is shown in Figure 1 AB. As can be seen H_2O_2 oxidizes a GP72-cysteine-Cys67 to disulfide bond. This can then either form an intra-molecular disulfide bond with Cys66 or interact with PDI directly. They further suggest that ERD1, PDI and GP72 form a triad which can utilize a single molecule of oxygen to produce two disulfide bonds and two molecules of H_2O (Wang, et al., 2014).

In addition to the evidence showing that GP72 and 8 are capable of directly oxidizing PDI to drive disulfide bond formation, evidence has also been presented to show that PDI44 has the capacity to directly oxidize PDI and drive disulfide bond formation in the presence of H_2O_2 (Thawker, et al., 2012). PDI44 is a member of the protein disulfide isomerase family. These proteins are known to regulate processes which are thought to allow secretion in *S. aureus* as it demonstrated that it was actually localized to the ER. PDI44 is considered a member of the type 2-Cysine (2-Cys) family of protein disulfide isomerase. In their study, the authors show that PDI44, like other members of this family, also contains an additional cysteine, known as the reducing cysteine, near its C-terminus. Catalytic activity of these 2-Cys protein disulfide isomerase is facilitated by the formation of mixed disulfide intermediates composed of 5 catalytic sites. Multiple formation provides the optimal arrangement of the peroxidatic cysteine to facilitate catalysis (Thawker, et al., 2012) (Wang, et al., 2012). PDI44 reacts in a two-stage of H_2O_2 in the ER (Wang, et al., 2012), where reaction with H_2O_2 oxidizes the peroxidatic cysteine to become oxidized (Figure 1 A). Subsequently causes a conformational change within the PDI44 dimer which brings the reducing cysteine in

expression of components of the ubiquitin-protein machinery (Petrovic & Kaufman, 2005). Over-expression of gp78 in rat indicated to control of the two. Langer et al found that gp78 mRNA, which correlated with actual protein expression, was highly

expressed in primary resected oesophageal adenocarcinoma tissue sections, particularly in early stage (McCaughey et al., 2008). There is also evidence that suggests that activation of Gadd153 by DNA damage and oxidative stress, sensitive cells to ER stress leads to a depletion of cellular glutathione (GSH) and increased production of ROS (McCaughey et al., 2009). Glutathione has an important role in redox protein folding, assisting PDI, in the reduction of the disulfide bonds of misfolded proteins (Chakravarti, et al., 2004). This evidence, taken together suggest that cellular injury, such as from the acid exposure of GERD, a known precursor of BC, results in the accumulation of misfolded proteins in the ER, disrupting cellular GSH and so leading to an increase in oxidative protein folding to completion.

Western Blotting of tissues of the adenocarcinoma cell line, OE23 compared to the equivalent cell carcinoma cell line OE21 also used a greater sensitivity to stress, induced by oxidative stress agents (DMSO, CuCl₂ and Tunicamycin), determined by an increase in ERD1α expression in the OE23 cell line compared to OE21 cells (Bialek et al., 2015). A natural consequence of the increase in expression of ERD1α would, therefore, be an increase in the generation of H₂O₂ (Figure 1A) as a by product. However, as can be seen in Figure 1A B and C both OE21 and OE23, misfolded in the ER can utilize H₂O₂ to both release ERD generated H₂O₂ and to increase protein folding. Rimmering et al showed that loss of GP48 resulted ER stress, leakage of H₂O₂ into the cytosol and cell death. They suggest that the ER resident GP48, particularly GP48, rather than PDI is responsible for the

production of H₂O₂ accumulation and damage from the ER and that there is only modest H₂O₂ detoxification when levels of ERCh collapsed H₂O₂ in mammalian (Rammung, et al., 2016). Further Peng et al demonstrated that GPX7 has the capacity to prevent oxidative DNA damage. They showed that GPX7 expressing cells had lower levels of 8-OHdG, 8-hydroxydeoxyguanosine ROS and oxidative DNA damage, in particular double strand breaks compared to controls when exposed to an oxidized bile acid cocktail (Peng, et al., 2015). That the protective ability of GPX7 and Prdx6 does not seem to occur suggests that either the production of H₂O₂ overwhelms the ability of GPX7 and Prdx6 or the antioxidant requires an optimal equilibrium of the antioxidant that disrupts or reduces their ability to utilize and reduce H₂O₂ resulting in its accumulation and in turn oxidative DNA damage. Some evidence for loss of H₂O₂ detoxification currently exists with respect to GPX7. Peng et al demonstrated that the GPX7 promoter was silenced by histone-specific methylation *in vitro* and *in vivo* mouse models of CRC. They further demonstrated that expression of GPX7 in these models led to a reduction in tumor growth and an increase in cell death/apoptosis and suggest that GPX7 may be a possible clinical therapeutic role in CRC (Peng, et al., 2016). The loss of GPX7 has been thought to play a major role in the pathogenesis of CRC, experiments have shown that loss of GPX7 can activate pro-inflammatory pathways through activation of NF- κ B via TNF- α induction (Peng, et al., 2014). The binding domains will not address the oxidative cell dysregulation, acid exposure resistance or apoptosis via NF- κ B activation and reduce ROS/RNS production and DNA damage, particularly double strand breaks in tissue biopsies from patients with biliary CRC (Jha, et al., 2011). Demethylating acid did not reduce DNA damage in colorectal NF- κ B in normal mucosal cells (Zhang, et al., 2008) (Jha, et al., 2011) suggesting that while loss of GPX7 may have a role in

the pathogenic transformation of SDC to DAC in vivo in the adult transformation of the oncogene to SDC's vehicle.

In addition to evidence linking SDC to and the loss of SDC with SDC and DAC, PDL has also been linked with progression in many cancers, such as breast and prostate carcinoma (Ruhle, et al., 2015), primary colon tumors (Seydel, et al., 2015), glioblastoma multiforme, an aggressive brain malignancy (Poh, et al., 2012) and oral cavity squamous cell carcinoma (Chang, et al., 2015). In particular PDL seems important in the metastasis of these cancers (Chang, et al., 2015) (Ruhle, et al., 2015) and in the silencing of integrin α 5 β 1 (Seydel, et al., 2015) (Poh, et al., 2012). In breast and prostate cancers, it has been observed, by mass spectrometry that perlecanidase was enriched from the cancer cells. A mass analysis of samples taken from breast and prostate cancer patients determined that the gene expression of PDL was increased in carcinoma tissues. It was also seen that patients who developed metastases had significantly increased expression levels of PDL than those who remained metastasis free after 5 years. The tumor is a common site of metastasis in breast and prostate cancer (Shen, et al., 2008) and in order to reduce tumor growth, growth factors and cell cycle inhibition cancer cells must not be inhibited. PDL and its associated perlecanidase and the cell line deficient in PDL demonstrated a decreased ability to stimulate cancer formation in vivo. Tumor mice, that injected with PDL cancer cells in which PDL had been knocked down displayed significantly smaller subcutaneous tumors and a decrease in collector number compared to control PDL cells (Ruhle, et al., 2015). A similar finding has also been found in oral cavity squamous cell carcinoma (OSCC) which is associated with a high probability of cervical lymph node metastases. It presented

analyzed by Cheng et al. revealed differential expression of Pfdel in comparing metastatic and primary OSCC tumors. Analyzed by immunohistochemistry revealed significantly ($P < 0.0001$) higher levels of Pfdel expression between tumor cells and neighboring epithelial cells and in 31 metastatic lymph node tumors compared to corresponding primary tumors (Cheng et al., 2011). In glioblastoma multiforme (GBM), an aggressive brain malignancy, Kim et al. found that Pfdel expression was upregulated in tumors and in mouse GBMs, using Pfdel expression with an increase in ROS. They detected an increase in ROS in mouse GBMs, compared to wild type controls suggesting that Pfdel promotes neural progenitor GBM growth. In order to test the hypothesis they used a lentiviral GFP tagged expression system to knockdown Pfdel expression in GBM neurospheres. This resulted in an increase in ROS accumulation. DNA damage, an increase in apoptosis and cancer cell reduction in GBM growth (Kim et al., 2012). This finding is supported by a small study in colon adenocarcinoma, where Pfdel was also found to be over expressed in tumor cells compared to normal colon tissue. Here knockdown of Pfdel gene expression resulted in an increase in apoptosis (Lambert et al., 2011). While this increase in apoptosis may be related to its antioxidant properties, there is evidence to suggest that over expression of Pfdel may actually inhibit part of the apoptotic pathway by downregulating TNF related apoptosis-inducing ligand (TRAIL) independent of ROS production (Hong et al., 2010). Taken together the evidence suggests a significant role for Pfdel in various disease cancers, it is therefore not a great leap to imagine that downregulation of Pfdel may have a role in CRC and therefore possibly in BC.

17 Author: Graham J. M. Orr, resident member of the F01 family which may have a role in the pathogenesis of Osteopetrosis Alveolar Osteitis.

Author: Graham J. M. Orr, resident member of the F01 super family (Preston, et al., 2005) it was first discovered in *Amphispiza bilineata* common gnatcatcher where it has a role in skeleton patterning (Steger, et al., 1995). In *Helio helios* AGS2 has a role in neural production, particularly the MLC2 neuron in the retina (Bergman, et al., 2014) (Pohl, et al., 2005) and the MUC1 and MUC5AC family members (Pohl, et al., 2015). Like other ER proteins AGS2 contains a C-terminal localization sequence (Figure 1.5), however rather than the usual KDEL, sequence (Blanc & Pichon, 1997), AGS2 contains a novel TEE sequence. Deletion of the motif results in AGS2 secretion and loss of AGS2 function (Gable, et al., 2015). Various studies have shown that while AGS2 exists intracellularly it can also be secreted (Gable, et al., 2015), which has important functions related to health and disease (Bergman, et al., 2014) (Pohl, et al., 2015). AGS2 contains a single transmembrane fold domain, comprising of a CysE motif rather than the classical CysC motif (Figure 1.5), suggesting the AGS2 may be a late evolving member of the F01 family (Bergman, et al., 2015) (Pohl, et al., 2005). Recent evidence suggests that AGS2 may form dimers through the single conserved CysC motif suggesting that dimerization may be required for disulphide exchange between AGS2 and its client proteins (Coker, et al., 2016). Experiments by Pohl et al have shown both that AGS2 interacts with SP-100/79 and the dimerization required for its function (Pohl, et al., 2015) although when other client proteins AGS2 interacts with and by what mechanism AGS2 facilitates disulphide exchange, if indeed that, is so far not clear.



Figure 1. Schematic diagram of the protein structure. The signal peptide is located at the N-terminus, and the C-terminal is located at the C-terminus. The protein is composed of two main domains, L1 and L2, and five loops, L3, L4, and L5.

1

Figure 2. Schematic diagram of the protein structure. The signal peptide is located at the N-terminus, and the C-terminal is located at the C-terminus. The protein is composed of two main domains, L1 and L2, and five loops, L3, L4, and L5.

Figure 3. Schematic diagram of the protein structure. The signal peptide is located at the N-terminus, and the C-terminal is located at the C-terminus. The protein is composed of two main domains, L1 and L2, and five loops, L3, L4, and L5.

Figure 4. Schematic diagram of the protein structure. The signal peptide is located at the N-terminus, and the C-terminal is located at the C-terminus. The protein is composed of two main domains, L1 and L2, and five loops, L3, L4, and L5.

Other than AGO2, which is involved in both formation and the maturing of these complex 19S glycoproteins, such as the major 19S2 (Pahl, et al., 2005) and MUC1 and MUC5AC (Pahl, et al., 2013) little seems to be known about AGO2's role in normal physiology. Of more importance is the role that AGO2 also has in disease. AGO2 appears to have roles in a number of cancers: it specifically promotes thyroid cancer and prostate cancer and is involved, particularly in gastric hormone independent cancers (Bryantsev, et al., 2011). As an ER related protein AGO2 has a role in the control of ER homeostasis and its expression is increased in control of the unfolded protein response in ER stress (Jha, et al., 2011). In cancer biology AGO2 seems to be involved in a number of processes relating to cell adhesion (Pahl, et al., 2013) metastasis in overexpression positive breast cancer (Fischer, et al., 2005) and as a tumor marker (Fischer, et al., 2006). Although in breast and prostate cancer increased AGO2 expression seems to be related to terminal silencing (Su, et al., 2012), there are several strong lines that suggest the ER stress plays an important role in AGO2 involvement in cancer. Experiments by Dumortier et al showed that in pancreatic ductal adenocarcinoma (PDAC) increased AGO2 expression was present in the early stages of neoplastic transformation and as such preceded the development of PDAC. They found that silencing subsequent to AGO2 expressing cells was present neighbouring pre-neoplastic lesions and that correlates with their position relative to neoplastic foci. The AGO2 was overexpressed in cancer stem cells compared to cancer cells of non stem origin. Further Dumortier et al use, in a mouse model, that AGO2 induction preceded the development and formation of pre-neoplastic lesions and that deletion of AGO2 expression inhibited tumor growth. They also showed that tamoxifen induced ER stress increased the expression of AGO2 and that the increased AGO2

expression in prostatic, oesophageal squamous carcinoma after treatment of the cells by genistein response was noticed by (Durbani, et al., 2016). The AGS2 expression is involved in neoplastic transformation in cancer although evidence suggest that AGS2 is increased from the ER (Guan, et al., 2012) and in the nucleus AGS2 may be responsible for strong transcriptional. It is supported by Fares et al provide some evidence for this, in their study they showed that AGS2 was present within the extracellular matrix. Duplication of AGS2 from a lung carcinoma cell line resulted in a loss of tumorigenicity and metastatic potential and that the addition of recombinant human AGS2 to the media of normal fibroblasts cells line depleted of AGS2 restored their ability to form tumour organoids. They also demonstrated the metastatic potential of AGS2. In vitro normal human bronchial epithelial cells (HBE) form polarized, spherical organoids with hollow lumens, addition of AGS2 to the extracellular matrix resulted in the formation of large non-polarized, unstructured organoids. Lacking a lumen. (Fares, et al., 2016) suggesting a possible mechanism where, if normally ER localized AGS2, was secreted it may be able to induce tumorigenesis.

Finally a role for AGS2 has been suggested as a tumour suppressor in cancer. Specifically in CRC. A study by Polkin et al showed that AGS2 was overexpressed in lysates of CO tissue in comparison to normal coloproctal tissue lysates, and that overexpression of AGS2 attenuated the phosphorylation of p53, a tumour suppressor protein in response to DNA related stress. However AGS2 does not seem to interact directly with p53, so the mechanism by which it suppresses p53 and p53 controlled apoptosis is as yet unclear (Polkin, et al., 2006). It is evident, nevertheless, that AGS2 appears to be heavily linked to the pathogenesis of a number of cancers via many different roles.

In summary, Barrett's Oesophagitis is a complex disease, related to the fact that the cells of the oesophagus are under constant environmental pressures. One that seems likely to be food related from chemicals and food products and from exposure to refluxed acid, pepsin and bile products. This scenario is exacerbated further in those that suffer from GERD. Such a complex mechanism may mean that the exact cause of the transition from BO to OAC is individual to each patient, however there are many possible targets for biomarker development that may aid in determining the risk of those presenting with Barrett's Oesophagitis transitioning to OAC.

1.8 Aims

There is evidence to suggest a link between the damage caused by ROS such as oxidants and oxygen radicals and BC. Cells and tissues have a number of antioxidant proteins and other molecules that prevent damage by ROS including glutathione peroxidase (GPx) and peroxiredoxin (Prdx). Accumulation of ROS may favour the evolution of BC to OAC. Recently, members of the GPa and Pdx families have been implicated in controlling protein folding and stress responses in the endoplasmic reticulum. However, the extent to which members of the GPa and Pdx families are dysregulated in BC and OAC is not known.

The aim of this thesis is to

- Determine the expression levels of the GPa family member GPx2 and the Pdx family member Prdx6.
- Expose the oesophageal cells to ER stress inducing agent and analyse the expression profiles of GPx2 and Prdx6.
- To relate any findings to the whole organ the expression of GPx2 and Prdx6 in normal oesophageal will be compared with tissue resection patients with BC and OAC and examined by immunohistochemistry using confocal microscopy.

Materials and

Methods

2.1 Antibodies

The following antisera were used: monoclonal anti-human IgG1 anti-Fc γ 2b (2H10) (54), and IgG2 γ (2H3) (57) were obtained from Abcam. A polyclonal anti-Fc γ 2b anti-human IgG1 was a gift from the Yang Group (Yang, et al., 2014) (55) and anti-human IgG2 γ were used the first IgG2 γ (2H17) was obtained from Cell Signalling and the second were a gift from Neil Butler (Butler & Jones, 2004). Polyclonal anti-human IgG1 anti-Fc γ 2b and CALS was a kind gift from the Chubb Group (Chubb, et al., 2011). Polyclonal anti-human IgG1 anti-Fc γ 2b, anti-human IgG1 anti-Fc γ 2b anti-Fc γ 2b and ER2 γ -L α (940) (955) were obtained from Santa Cruz Biotechnology. Monoclonal anti-human IgG2 γ was obtained from Cell Signalling. Human IgG1 anti-Fc γ 2b (M3-101) was obtained from Biologics and anti-human IgG2 γ was a kind gift from Michael Lee (Cheuk, et al., 1997).

2.2 Cell Lines

The following commercial, immortalised cell lines were used: H7150 (5) (Boulikas) a fibrosarcoma cell line, originally obtained from fibrosarcoma tissue taken by biopsy from a 50-year-old female (Boulikas & 1979). Hs578t (56) derived from serous ovarian carcinoma (Schwarz W, 1975). OE19 (56) (71) (Kuo) was derived from an Adrenocortical carcinoma taken from a 72-year-old male, OE21 (56) (22), (Kuo) derived from a Squamous carcinoma taken from a 68-year-old male, and OE33 (56) (68), (Kuo) derived from an Adrenocortical carcinoma taken from a 73-year-old female (Boulikas, 1987).

2.3 Cell Culture

HT1080 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco-ThermoFisher Scientific). HeLa cells were maintained in Minimum Essential Medium (MEM; Gibco-ThermoFisher Scientific) and OE19 and OE23 cells were maintained in Roswell Park Memorial Institute Medium (RPMI 1640; Gibco-ThermoFisher Scientific) all supplemented with 10% Fetal Bovine Serum (FBS; Sigma) and glutamine (Invitrogen) 100 units/ml penicillin and 100 units/ml streptomycin (Invitrogen) at 37°C and 5% CO₂. All cell lines were serially passaged at 70-80% confluency using 0.05% trypsin (Trypsin-EDTA, Invitrogen) to digest the monolayer before resuspending in fresh media. OE19 and OE23 cells were typically split 1:1 into T25 flasks (TPP Helena Biosciences) and the OE cell lines were added 2.5 into T25 flasks (TPP Helena Biosciences). All cell cultures were kept in an incubator (Gibco) in humid conditions in 5% CO₂ (low pressure) at 37°C.

2.4 Transfection

OptiTM (Miyoshi Chemicals, Japan) and the DCl₃ in water (Sigma) solution, combined to give electroporation buffer (EOP buffer; Shimizu, et al., 2002) using a Stratagene gene charge kit were used for the transfection of HeLa cells. The HeLa cells were cultured in 60-cm culture dishes, containing 3 × 10⁸ cells, in 60% confluency in Minimum Essential Medium (MEM; Gibco-ThermoFisher Scientific). The cells were transfected using Lipofectamine 2000 (Invitrogen, ThermoFisher). A master mix was prepared for each cDNA to be transfected. For the OptiTM master mix 2 μg OptiTM cDNA and 4 μl 2000 were added to 150 μl Opti-MEM (Gibco-ThermoFisher Scientific) and for the DCl₃ in water cDNA cDNA master mix

1 µg of CEM4-CDNA and 4 µg/2000 were added to 150 µg of cells. The translation signal was prepared in parallel tubes each containing 15 µg of Qse-MEM and 7.5 µg of Lipofectamine 3000 and an equal amount of each 2-CDNA master mix was then added to each tube which were then left for 5 minutes to incubate before adding to the 80% confluent HeLa cells, which were washed twice with 1 ml sterile PBS before the transfection reagents were added. Each dish was then left to incubate at 37°C, 5% CO₂ for 2 hours after which 1 ml of MEM was then added to each dish, then left overnight to reach full confluence before analysing by immunofluorescence.

23 Cell Lysate Generation

Cells were lysed in lysis buffer (1 mM dithiothreitol buffered saline (PBS, Sigma) with 1% Triton X-100 (Sigma), supplemented with 10 µg/ml each of chymotrypsin, leupeptin, aprotinin and pepstatin (ECLAP) and 20 mM Na-orthovanadate (NEM) when required. Nuclei were removed by centrifugation at 10,000 g (5 minutes) for 10 minutes at 4°C and the Post-nuclear supernatant collected. Both fractions liquid nitrogen and stored at -80°C until analysed by SDS-PAGE.

24 Cell Lysate Protein Quantification

A Pierce™ BCA Protein Assay Kit (Thermo Scientific) was used to determine the protein concentration. The assay kit is a colorimetric detection assay based on the biuret reaction. The reduction of Cu²⁺ to Cu⁺ with detection and quantification of total protein using a nitrogen-containing chromophore and BCA. The procedure is

purple coloration reaction products formed by the oxidation of free molecules of BCA to the cuprous ion. This is why a strong absorbance at 562nm due to color is seen over a protein concentration range of 20-2000µg/mL. BSA standards from 25µg/mL and 2000µg/mL obtained in lysis buffer (minus CLAP and with or without NEM, sample absorbance were prepared in order to generate a standard curve. Then 0.025mL of the lysis and each standard were combined with 1mL of the kit working reagent in separate tubes (6-pipette) and incubated in a water bath at 37°C for 30 minutes, the tubes were then cooled to room temperature and read on a spectrophotometer (Genios®) at 562nm. Absorbance were read within 10 minutes as the BCA assay has no true endpoint and so the colour will develop further over time. The standard curve was drawn and lysate concentration determined using Microsoft Excel.

2.7 Immunoprecipitation

Protein A sepharose beads (Sigma) were prepared by eluting 1.10g of bead slurry in lysis buffer. The beads were spun at 1500g, 5 minutes, 4°C in lysis buffer (minus NEM or CLAP). Then the supernatant was aspirated off and the beads were resuspended in fresh lysis buffer and spun again at 1500g, 5 minutes, 4°C. The supernatant was discarded and the beads were resuspended in 1mL of antibody in lysis buffer and incubated for 2 hours at 4°C with rocking. After incubation the beads were spun at 1500g, 5 minutes, 4°C, discarding supernatant, resuspended in lysis buffer, then spun again at 1500g, 5 minutes, 4°C. The supernatant was discarded and the beads were then resuspended in 1.4 volume of lysis buffer in lysis buffer. This incubated for 1 hour at 4°C with rocking. The bead on 8 minutes was then spun

cells at 100% 5 minutes, 4% - measuring expression and measuring in both dish before being spun. Finally the supernatant was removed off and the total cell mix was resuspended in 200ul DTT (2x barford) sample buffer (Bio-PAGE LDS sample buffer, Invitrogen), heated at 95°C for 5 mins, then analysed by SDS PAGE (method 2.16).

2.8.1. Immunoblot

6 control dishes in 6 wells change in protein expression was either dependent or independent, CE19 cells were treated with either under reducing or oxidising conditions. To examine the effects of reducing conditions on protein expression CE19 cells were cultured in three 6cm dishes in confidence. The first dish was kept as a control, the culture media was removed off and fresh RPMI was added and the cells were incubated at 37°C 5% CO₂ for 5 minutes. The cells were then washed once for 5 minutes with PBS with 20mM NEM, then washed once more for 5 minutes with just PBS before being lysed. For dish two the media was aspirated and RPMI supplemented with 20mM DTT was added, the cells were then incubated for 5 minutes then washed once for 5 minutes with PBS with 20mM NEM, then washed once more with just PBS for 5 minutes before being lysed. The third dish was incubated and RPMI supplemented with 20mM DTT was added, the cells were then incubated for 5 minutes. The supplemented media was aspirated off and fresh un-supplemented RPMI was added and the cells were incubated for a further 10 minutes before being washed once for 5 minutes with PBS with 20mM NEM, then washed once more with just PBS for 5 minutes before being lysed.

To examine the effects of oxidising conditions on protein expression CE19 cells were cultured in three 6cm dishes parallel to the 6 control. The first dish was kept as a

control. The culture media was aspirated off and fresh RPMI was added and the cells were incubated at 37°C in 5% CO₂ for 5 minutes. The cells were then washed once for 5 minutes with PBS with 20mM NEM, then washed once more for 5 minutes with just PBS before being lysed. For each test the media was aspirated and RPMI supplemented with 5mM Chlorine was added. The cells were then incubated for 5 minutes then washed, once for 5 minutes with PBS with 20mM NEM, then washed once more with just PBS for 5 minutes before being lysed. The Bradford was aspirated and RPMI supplemented with 5mM Chlorine was added. The cells were then incubated for 5 minutes. The experimental media was aspirated off and fresh, un-supplemented RPMI was added and the cells were incubated for a further 10 minutes before being washed, once for 5 minutes with PBS with 20mM NEM, then washed once more with just PBS for 5 minutes before being lysed.

2.8 Immunofluorescence

2.8.1 Standard Protocol

A 24 well plate was prepared by the addition of a 10mM (pH 7.4) HEPES to each well of the well. The wells were then washed with 500µl PBS. Cells were cultured to 70-80% confluency then detached using 0.05% trypsin (Trypsin-EDTA, Invitrogen), diluted 1 in 10 with the appropriate media. 500µl of cell suspension was added to each well containing a coverslip and then incubated overnight at 37°C in 5% CO₂ to allow the cells to adhere to the coverslip.

The media was removed and discarded and the coverslips were washed twice with 500µl PBS + PBS containing 1mM MgCl₂ and 0.5mM CaCl₂. The cells were then fixed in 500µl 4% Paraformaldehyde (PFA) fixed from 10% stock in PBS) for 10 minutes. The cells were then washed twice with 500µl PBS+ before the antibody

groups were then quenched in 500µl 0.04M Na₂S₂O₄ for 10 minutes then washed three times in 500µl PBS⁺⁺. The cells were permeabilized in 500µl 0.1% Triton X-100 in PBS⁺⁺ for exactly 10 minutes, then washed in 500µl PBS⁺⁺ for 5 minutes, then blocked by washing in twice in 500µl BSA (Sigma) in PBS⁺⁺.

The blocking solution was fully removed and either 20µl Control (0.2% BSA in PBS⁺⁺) or 200µl of the appropriate primary antibody in a mix of primary antibodies from two different host animal species, diluted in PBS⁺⁺ according to Table 2.1, was added to each coverslip. The plate was then covered and the cells incubated at room temperature for 30 minutes, washed three times 5 minutes with 200µl PBS⁺⁺ before incubating in 200µl of the appropriate secondary antibody (Table 2.1), diluted 1:1000 in PBS⁺⁺ for 20 minutes in a dark dish.

The coverslips were then washed twice times 5 minutes in 500µl 0.2% PBS⁺⁺, once for 5 minutes in 500µl PBS⁺⁺ and then for 5 minutes in 40µl 1:1000 DAPI in PBS⁺⁺ all in the dark. The coverslips were washed a final time in 40µl 0.1% primary antibody or both the secondary antibody was removed and mounted, cell side down, onto a labelled microscope slide using hard set Vectashield. The slides were left, oriented on the bench for 20 minutes then transferred to 4°C if not before being examined on a Zeiss Axiovert microscope.

2.3.2 Immunofluorescence of Aβ₄₂ with 3B1 and 7E8 antibodies

2.3x and 3x plate were prepared by the addition of 10µl of cells per coverslip to each well to be used. The wells were then washed with 500µl PBS. Cells were culture to 70-80% confluency then detached using 0.05% trypsin (Tgptsin-CST, Invitrogen), diluted 1 in 10 with the appropriate media. 500µl of cell suspension was added to

cells were continuing to grow and then incubated overnight at 37°C, 5% CO₂ to allow the cells to adhere to the coverslip.

The media was removed and discarded and the coverslips were washed twice with 500µl PBS+ (PBS containing 10mM MgCl₂ and 0.5mM CaCl₂). The cells were then fixed in 500µl 4% Paraformaldehyde (diluted from 16% stock in PBS) for 10 minutes, then washed three times with 500µl PBS+. The coverslips were then covered with 500µl of 0.05M BSA and incubated at 25°C for 10 minutes, the coverslip were then fixed once in 500µl PBS+ for 5 minutes.

The cells were then blocked for 1 hour in 500µl Blocking buffer (5% normal goat serum, 0.2% Tween X-100 in PBS+). The blocking buffer was replaced off then 250µl of primary antibody, diluted according to Table 2.1, in PBS+. 0.2% Tween X-100 in PBS+ was added to each coverslip and incubated overnight at 4°C. After primary antibody incubation the coverslips were washed three times for 5 minutes in PBS+ before incubating in 250µl of the appropriate secondary antibody (Table 2.1), diluted 1:1000 in PBS+ for 20 minutes in the dark.

The coverslips were then washed twice times 5 minutes in 500µl 0.2% PBS+ once for 5 minutes in 500µl PBS+ and then for 5 minutes in 500µl 1:1000 DAPI in PBS+ all in the dark. The coverslips were mounted a final time in DAPI in presence and absence of BSA to check coverslip was removed and incubated, left side shows cells in labelled microscope slide using field see (unlabelled). The slides were left overnight in the dark for 20 minutes then transferred to 4°C till use before being examined on a Zeiss Axiovert microscope.

Primary Antibody	Host Species	Isotype	Secondary
1244	Mouse	1:200	Anti-Rat IgG
SP2	Mouse	1:200	Anti-Rat IgG
SP2	Mouse	1:200	Anti-Rat IgG
4957	Mouse	1:500	Anti-Rat IgG
791	Mouse	1:500	Anti-Rat IgG
791	Mouse	1:200	Anti-Rat IgG
1240	Mouse	1:200	Anti-Rat IgG
1242	Mouse	1:500	Anti-Rat IgG
4959	Mouse	1:500	Anti-Rat IgG

Note: 12. A list of Primary antibodies used in Immunofluorescence, electron beam systems, electron and scanning secondary Ab and Run (ThermoFisher)

2.16 SDS-PAGE

Protein nuclear supernatants and immunoprecipitates were taken up in 2x Laemmli sample buffer (BioRad) (SDS sample buffer). Intact oligos, heated at 95°C for 5 min, were analyzed by SDS-PAGE.

Samples were run on an acrylamide gel containing SDS which contains 0.1% Acrylamide (Sarens Biochem) percentage dependent on the molecular weight of the protein under investigation. 0.75M Tris (pH 8.8), 0.1% SDS (Sigma), 0.1% APS (Sigma) and 0.05% TEMED (Sigma). The running gel was run in the "buffer" gel color system, and polyacrylamide (PA), the running gel was covered with 0.02% to

ensure that the interface between the running gel and the stacking gel was smooth. Once polymerized the dH₂O was removed and the stacking gel was added, along with a 10 or 15 well comb. The stacking gel was composed of 5% Acrylamide (Green Biochem), 0.05 M Tris (pH 8.8), 0.1% SDS (Sigma), 0.02% APS (Sigma) and 0.02% TEMED (Sigma). After the stacking gel had polymerized the gel was transferred to a empty small 8 inch vertical electrophoresis unit and diluted 1 x TGS electrophoresis running buffer (10x TGS running buffer (BioRad) diluted with dH₂O) to cover the gel. The combs were removed and samples were loaded (approximately 2 µl of a known molecular weight protein marker (BioRad) 10 samples were equalized to 1/10th of protein per well) and analyzed under either reducing conditions or reducing conditions, with the addition of 50mM DTT to the sample buffer and run at 200V until the dye front reached the bottom of the gel, without running off. Then the gel was either Western Blotted or Stained with Coomassie Blue.

3.11 Gel Staining

The gel was fixed in 50mM fixing solution (7% Acetic Acid, 40% Methanol in dH₂O) for 10 minutes. The solution was removed and the gel was covered in Coomassie stain (20% Methanol and 80% Brilliant Blue G-coomassie concentrate (ThermoFisher). The gel was left to stain overnight at room temperature in a dark. The methanol was decanted and 50mM of destain solution (20% Methanol, 10% Acetic Acid in dH₂O) for 10 minutes before removing and destaining a second time, with 50mM 20% Methanol in dH₂O overnight, while rocking. The destain was then removed and the gel was washed in dH₂O and then imaged on a gel documentation system.

2.12 Western Blotting

Proteins separated by SDS-PAGE were transferred onto gel with a PVDF membrane (Millipore) in transfer buffer (25 mM Tris-HCl, 190 mM Glycine in 20% methanol) at 100 mA for 2 hrs or overnight at 4°C. Prior to use in transfer to PVDF membrane was pinned in methanol for 20 seconds. Membranes were then blocked in 1% BSA in TBS-T (20 mM Tris-HCl, 138 mM NaCl, 0.1% Tween-20, pH 7.4) either overnight at 4°C or 1 hr at room temperature, with rocking. Membranes were then washed three times, for 5 minutes each, with TBS-T, before being incubated in the primary antibody (in 1% BSA-TBS-T) either overnight at 4°C or for 1 hr, with rocking. Table 2.1 lists the primary antibodies used, their host species and corresponding dilutions. Membranes were rinsed in TBS-T, then washed 4 times, for 5 minutes each, in TBS-T, after which, the membranes were incubated with a secondary antibody (DABC) corresponding to the host animal species (see Table 2.1), diluted 1:2000 in 0.1% BSA-TBS-T for 1 hr, rinsed in TBS-T, then washed 4 times, for 5 minutes each, in TBS-T then once, for 5 minutes, in TBS. DAPI (4',6-diamidino-2-phenylindole) was then added to the membrane before it was exposed to film (ThermoFisher) then developed in an x-ray developer (D19A7).

Primary Antibody	Host Species	Secondary	Dilution
CD44	Mouse	GARFPO	1:1000
GFAP	Mouse	GARFPO	1:1000
GFAP	Mouse	GARFPO	1:1000
MAP2	Mouse	GARFPO	1:1000
PSD	Mouse	GARFPO	1:1000
CAHA	Mouse	GARFPO	1:1000
CTSL	Mouse	GARFPO	1:1000
MSD	Mouse	GARFPO	1:1000

Table 4.1. A list of primary antibodies used in Western Blotting. Including host animal, species, application, secondary and dilution. (Continued)

2.13 Molecular Biology

2.13.1 Transfection of DMS cells

DMS competent cells were transfected with mouse GFAP (Myc-DD) tagged in pCMV6. Figure 2.11. cDNA (Original) for mini prep. For each transfection 1µl GFAP (20µg) was heat shocked at 42°C for 1 minute, added to 50µl of DMS competent cells, then incubated at 37°C for another minute. LB broth (100µl, 10g Tryptone, 5g Yeast extract, 10g NaCl made up to 100ml), was then added for a total final volume of 1ml and incubated at 37°C for 45 minutes then centrifuged at 12000 rpm for 1 minute. Supernatant was removed and the cell pellet was re-suspended in 100µl cell lysis reagent with 0.25mg/ml Kanamycin and spread on LB-agar (100µl) plates, supplemented with 0.25mg/ml Kanamycin, overnight at 37°C. Single colonies were picked and incubated overnight at 37°C, with constant agitation. 100 µl of LB broth supplemented with 0.25mg/ml

columns were placed in the microcentrifuge tubes then left to stand for 15 minutes and then spun at 17,000g for 40 seconds. DNA concentration was determined on a LabTech NanoDrop spectrophotometer.

2.13.3 Gel Electrophoresis

Agarose Gel Electrophoresis was used to identify the presence of GP-IV DNA in samples from Mice Prap. A 1% agarose gel was used to run the DNA samples, the gel was made from 1.2g Agarose (BioLabs), 5.5ml ethidium bromide (Fisher) and 120ml 0.5x TAE buffer, diluted from a stock 50x stock solution (50.5g in 500ml 14.3M glycine sodium acid, 250 µl 0.5M EDTA made up to 250ml with dH₂O), which was then loaded with 10µl of sample containing either 1µl H9c2 Luciferase (BioLabs), 10µl pGL3-TOP, and 9µl Chosen free H₂O, 10µl free prep control, 1µl loading buffer and 9µl Chosen free H₂O or a vehicle sample of 1µl GP-IV DNA, 1µl loading buffer and 9µl Chosen free H₂O. The gel was submerged in TAE buffer and the samples were run at 100v for 20 minutes before visualizing under UV.

2.14 Tissues

Human Pancreatic Tissue was obtained from National Solutions P.L.C. Normal Cholangitis, Chronic Biliary Cholangitis and Cholangiocarcinoma Junction Tumor Tissue was provided by material transfer agreement from the Department of Histology at James Cook University Hospital.

2.15 Immunohistochemistry of human tissue sections

Paraffin-embedded tissue was cut to 4-µm sections using a microtome. The sections were floated in a 37°C water bath for a few minutes only, then floated sections were placed out of the water using polyamine coated slides and left to dry overnight at 40°C overnight.

2.15.1 Tissue Fixation Method

The slides were incubated at 37°C overnight prior to mounting them back into a Thermo Scientific Sequenza Manual Sliding Rack. Each reaction was washed sequentially with 100µl the following washes, 7 minutes each in Histoclear (Agar Scientific), a second 7 minutes wash in Histoclear, a 7 minutes wash in 100% ethanol, a 5 minutes wash in 50% ethanol, a 5 minutes wash in 70% ethanol and finally a 3 minutes wash in tap water. Each reaction was washed for 15 minutes in 2% H₂O₂ in methanol to block endogenous peroxidase activity.

Slides were then transferred to a rack for antigen recovery and incubated in 10mM sodium citrate buffer (pH 4.0) for 20 minutes at 95°C, were then, then cooled down and in room temperature. Sections were rinsed with a hydrophobic pen (from Edge Pen, Vector Laboratories H-4000) and 100µl 0.2% BSA in PBS was added to each reaction for 5 minutes. Sections were then blocked for 30 minutes, in 100µl 0.2% PBS plus 1% Normal Goat Serum (Dako) then incubated for 1h with 100µl primary antibody (antibodies used were the same as those listed in Tables 2.1 and 2.2) diluted in 0.2% PBS plus 0% Normal Goat Serum. Sections were then washed for 5 minutes with 100µl 0.2% BSA in PBS before incubation for 45 minutes with 100µl 1% Reagent C (Dako) in PBS. Reagent C solution was removed.

by dipping the slides and coating the edge of each 75mm section with formalin then washed for 5 minutes with 100µl 2% SDS. Slides were incubated for 30 minutes with 100µl 1% Reagent A + 1% Reagent B in PBS, prepared 15 minutes prior to incubation (PANG & FORD), then washed for 5 minutes in PBS. DAB solution was prepared by dissolving 10µg 3,3'-Diaminobenzidine and 1 DAB tablet (both Sigma) in 10ml PBS. Five 100µl of DAB peroxidase solution was added to each slide and left for 4 minutes, in the dark for 4 minutes, then rinsed in tap water. Slides were transferred to a slide rack and immersed in Harris Haematoxylin (Sigma) for 4 minutes, then transferred to tap water for 5 minutes. The slides were then transferred back to the Distammon Sliding rack. The sections were rinsed in 2 tap washes of 100µl 1% Citric HCL in Methanol. Then washed twice in tap washes using 100µl tap water. The sections were then washed sequentially with the following washes in their wash basin in 100µl 70% ethanol, tap washed twice in 100µl 70% ethanol, washed for 5 minutes in 100µl 100% ethanol then washed twice for 7 minutes in 100µl Histoclear. The sections were then mounted in DPX mounting medium and left to dry before examination under a Nikon microscope with a Nikon camera (Canon CRX 5 Day wireless camera).

2.1.2 Adapted Staining Method

The slides were incubated in DPX overnight prior to mounting then loaded into a Thermo Scientific Sequenza Manual Sliding Rack. Each reaction was washed sequentially with 100µl the following washes, 7 minute wash in Histoclear (Agar Scientific), a second 7 minute wash in Histoclear, a 7 minute wash in 100% ethanol, a 5 minute wash in 50% ethanol, a 5 minute wash in 70% ethanol and finally a 3

slides were then transferred to a rack for antigen recovery and incubated in 10mM sodium citrate buffer (pH 6.0) for 20 minutes at a 95°C waterbath, then cooled down on ice to room temperature. Sections were rinsed with a Tris/NaCl/BSA (pH 7.4) (Edge Plus, Vector Laboratories #4033) and 100.0µL 2% BSA in PBS was added to each section for 5 minutes. Sections were then rinsed using the Vector Laboratories RTU reactivation universal Quik Kit (PK-7300). The sections were transferred to a moist chamber and blocked in 100µl prediluted blocking serum (2.5% Normal Horse Serum for 10 minutes, excess serum was then diluted from sections which were then washed for 5 minutes in 100µl PBS. Sections were incubated in 100µl of the appropriate primary antibody (antibodies used were the same as those listed in Tables 2.1 and 2.2), diluted 1:200 in 0.2% BSA in PBS containing 1% prediluted blocking serum for 1h. The sections were then washed for 5 minutes in 100µl PBS. Following this the sections were then incubated in one drop (1-20µl) of prediluted biotinylated pan-specific secondary antibody for 10 minutes before washing for 5 minutes in 100µl PBS and then incubated in one drop of RTU streptavidin-peroxidase complex reagent for 5 minutes. Sections were washed for 5 minutes in 100µl PBS and were incubated in 100µl of DAB-peroxidase substrate (Vector Laboratories SK-4105 consisting of 3.0µl DAB-peroxidase chromogen in 100µl DAB-Substrate for 4 minutes in dark or until desired intensity developed. Then rinsed in 100µl tap water. Slides were transferred to a slide rack and immersed in Harris Haematoxylin (Sigma) for 4 minutes, then transferred to tap water for 5 minutes. The slides were then transferred back to the Separators Sliding rack. The sections were rinsed in 3 liter washes of 100µl 7% Citric Acid in Tris.

sections were washed twice in each solution using 100µl (200µl for other substrates) of 0.1M NaHCO₃ (pH 10.5) MgSO₄ and rinsed in 100µl (200µl) of 0.1M NaHCO₃. The sections were then washed sequentially with the following washes: a fast wash twice in 100µl 70% ethanol, fast washed twice in 100µl 90% ethanol, washed for 5 minutes in 100µl 100% ethanol, then washed twice for 7 minutes in 100µl HistoClean. The sections were then mounted in DPX mounting medium and left to dry before examination under a Nikon microscope with a live recorded Camera CME3. Snap wireless camera.

**Antibody Testing
and Method
Evaluation**

2.1 Introduction

Immunofluorescence (IFC) is the combination of anatomical, pathological and biochemical techniques to identify specific tissue components using the interaction of target antigens (from a protein) with an appropriately tagged antibody. Before embarking on the staining of the human patient samples, which were kindly provided by James Cook University Hospital, it was important to outline the IFC method to ensure the best consistency of results. In principle the IFC method consists of a number of discrete steps including sample preparation, tissue permeabilisation and antigen retrieval, staining, mounting and finally visualisation. In order to optimise these steps a series of experiments using commercially available pancreatic tissue was conducted to verify work using the linked patient samples.

In addition, some antibodies including those raised against cathepsin (CA9) and calretinin (CALR), which had been provided as gifts from the Clarke group (see Chapter 2.1), had not been tested on human tissues and their efficacy in immunofluorescence and Western blotting (of human cell lines) had yet to be defined.

2.1.1 Histology of the Pancreas

The majority of the human pancreas consists of exocrine tissue, arranged in lobules comprised of pancreatic acinar cells and ductal tissue which drains from the main pancreatic duct into the common bile duct (Sharma, 2004). Pancreatic acinar cells store a zymogen granule which is released into the small intestine. The role of the acinar cells, of the exocrine pancreas, is in the production and

secretion of the production of a wide range of enzymes. At each site, cells with different ER biogenesis pathways (to the basal part of the cell). In contrast, the apical part of the cell can contain a variable number of vesicles/lysosomes containing pre-synthesized enzymes depending on where the cell is in its secretory cycle (Shewars & Laver, 1997).

In addition to the vesicles produced from the pancreas also contain endocrine-like insulin (beta cells) and glucagon (alpha cells) throughout the main exocrine tissue, known as the islets of Langerhans. The islets of Langerhans are composed of many cell types including β cells, which secrete insulin, α cells which secrete glucagon and D cells which secrete somatostatin. The islets do not connect to the pancreatic duct system but instead secrete directly into the bloodstream via an extensive capillary network of blood vessels (Walter, 2004).

3.1.2 The role of the Calnexin and Caliculin in the Endoplasmic reticulum.

Calnexin (CANX) and caliculin (CALR) are ER resident chaperones, part of the main family of proteins. Along with BiP, a protein disulfide isomerase and members of the PD family (Ergard & Finkel, 2003). CANX and CALR interact with and aid in protein folding and disulfide bond formation of newly synthesized N-linked monoglucosylated glycoproteins that have undergone partial trimming of the core fucose (GlcNAc)₂Man₅ which is added at the formation of monoglucosylated glycoproteins via a side chain containing an Asparagine-N-Glycanase sequence during translocation into the ER (Helenius, et al., 1997) (Ergard & Finkel, 2003). CANX is a 68 kDa non-glycosylated type 1 membrane protein consisting of 726 amino acids with 10 disulfide bonds located at the luminal side of the ER. It consists of homologous CALR is a 68 kDa soluble protein of 606aa.

CALP has a KDEL ER retrieval sequence and is oriented apical with a high signal to charge. It might be responsible for ER turnover and Ca^{2+} loading (Friedrich, et al., 1997).

As has been briefly discussed in the introduction, ER resident dependent and components of the calnexin protein folding machinery may have some role in the progression of breast Cancer (Peters, 2005). In Developmental Adenocarcinoma (DAC) While there is no evidence to suggest that ERG1, CANX and CALR are involved in DAC there are suggested links that involve ERG1 and CANX in other cancers. Dixon and et al demonstrated that, in human melanoma metastasis, there was a differential down regulation when comparing CANX and CALR. They found that CANX but not CALR was down regulated in melanoma metastasis, although they also observed that the downregulation, while being statistically significant was not absolute, in that it was only down-regulated in approximately half of the metastatic cells (Dixon and et al., 2004). In contrast up regulation of CALR expression has been found in human ductal breast carcinoma (Kobayashi, et al., 2012) (Dimitrakis, et al., 2016) where it has been suggested that the up regulation may have an effect on the ERK1/2 pathway (Dimitrakis, et al., 2016). Finally there is also a suggestion that protein or that loss of ERG1 is representative of the presence of malignant squamous cell carcinoma (SSCC) may contribute to tumorigenesis (Chen, et al., 2014). This evidence, when together, suggested that it was worthwhile to test the efficacy of the CANX and CALR antibodies, in range of techniques to be used prior to commencement of the main body of experiments.

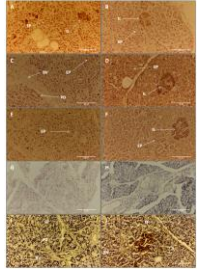


Figure 1. Micrographs showing the morphology of the cells and tissues at different stages of the experiment. The images are arranged in a 4x3 grid. The top three rows show brownish, textured surfaces, possibly representing different stages of cell culture or tissue development. The bottom row shows a more complex, fibrous structure, possibly representing a different tissue type or a specific stage of differentiation.

3.2 Results

3.2.1 Initial tissue staining experiments and method evaluations.

Initial staining of paraffin was carried out using commercially sourced pancreatic tissue stained with the anti insulin IHC antibody as an indicator of Langerhans isletlet and a measure of success, using procedures optimised by Gustavsson et al (Gustavsson, 2006). As can be seen in Figures 3.1 A and B the IHC staining was strongest in the areas of Langerhans, as expected, for the 100x visible view, whilst throughout the tissue. Besides some background was visible around the islets of Langerhans further experiments were carried out varying the IHC development time. For the second set of experiments the IHC incubation time was increased from a control of 5 minutes (Figures 3.1C and D) and 7 minutes (Figures 3.1E and F), respectively and the IHC antibody (Figures 3.1C and F) was included alongside a negative control (Figures 3.1C and E). There was little difference in the intensity of the IHC staining between the 5 minute IHC development, IHC control, pancreatic tissue (Figures 3.1C and F) versus IHC development, IHC control, pancreatic tissue (Figures 3.1E). However there appeared, in the control tissue (Figures 3.1C and E) a loss of the stain arising from the Haematoxylin counterstain. Based on these observations a new primary antibody was used at 100x magnification and which was specific for IHC development, although with the caveat that the result was only demonstrated using the anti insulin IHC antibody on human pancreatic tissue and may prove to be primary or secondary antibody dependent and/or tissue specific.

Of additional note in Figure 3 was the apparent lack of any visible counterstain from the Haematoxylin stain (see Chapter 2, 2.1.1). Since the stain nuclear stain from haematoxylin is pH dependent, its intensity could be increased by substituting

Scott's Top Water Solution system of normal top water (Brown, 2002). To test the concept, hair was cleaned with benzene before being dried in either top water (Figure 3.10) or a solution of Scott's top water (Figure 3.10). From this experiment it was apparent that Scott's top water gave a better staining result and so this was then further studied when co-staining with DNA. Fluorescent hair was then stained with the H&E antibody, in a positive control (Figure 3.11) or without H&E (Figure 3.12) as a negative control and compared. As can be seen in Figure 3.11 and 3.12 the use of Scott's top water gave much better contrast than those previously stained with top water (Figure 3.14-15).

3.3.2 CALB is expressed strongly in all cell lines and exhibits a staining pattern which suggests that it is predominantly ER localized

Polyclonal antibodies raised in Rabbit against CALB and CABC (see Chapter 2 section 2.1) were tested in immunofluorescence. CALB is an ER localized protein, in order to validate the specificity of CALB in immunofluorescence the cell line BT20 was incubated with CALB as per the method previously (see Chapter 2, section 2.3.1). Additionally three different CALB antibody dilutions were tested against a 1:200 dilution of FITC as a positive control.

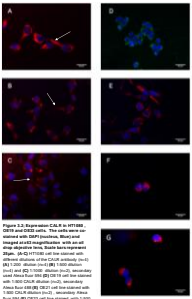


Figure 3. Immunofluorescence (IF) of cells stained with DAPI (blue) and other markers (red and green). Scale bars are shown in the bottom right of each panel.

and colocalized with DAPI, a marker of the nucleus (Figure 3.2 A-C and G). FOCI show the classical staining attributed to the ER of Figure 3.2G surrounding the DAPI stained nucleus, stained in blue with the FOCI localized in the ER seen primarily to one side of the cell. Given that the ER is a continuous membrane system interconnected with the nuclear membrane (Schmitt & Blobel, 2016) this pattern of expression is as expected and demonstrates of ER specific staining. In comparison the expression pattern of the CALR is very similar (Figure 3.2A-C) with a clearly defined ER with increased staining localized to one side in a similar manner to that observed in FOCI stained HT1080 cells (Figure 3.2G). These data however appears to be some staining that may be cytoplasmic (indicated by arrow (Figure 3.2A-C)). The second purpose of this experiment was to determine the location of the antibody at which consistent results could be observed. These dilutions were initially selected: 1:200 (Figure 3.2A), 1:500 (Figure 3.2B) and 1:1000 (Figure 3.2C). As can be seen in Figure 3.2(A-C) as antibody dilution produced the expected staining pattern. At 1:200 dilution (Figure 3.2A) the staining appears very intense and there doesn't appear to be any non-specific staining. A similar trend was observed with the 1:500 dilution (Figure 3.2B). The expression pattern was still clear although it appeared less intense. As an antibody dilution of 1:1000 (Figure 3.2C), the expected ER staining pattern was visible however the results were not consistent across the slide with some cells being more intensely stained than others. Taken together this suggest that a dilution of 1:500 provides a consistent result for use in further experiments. Finally, as osteopetrotic cell lines were the primary cell type to be used in the experiments it was also important to test if similar

antibody could be obtained with the immunoprecipitated cell lines to be tested. OEC19 (Figure 3.10), OEC21 (Figure 3.11) and OEC33 (Figure 3.12) were also tested with a 1:500 dilution of the antibody against CALR and compared against FOD (FOD replicates not shown). All these immunoprecipitated cell lines showed a similar expression pattern to that obtained with the H7100 cells and the same corresponds to that of FOD in the same cell lines, demonstrating that the CALR antibody appeared to detect CALR consistently across multiple cell lines. However, it was noted that some of the staining may extend beyond the ER (Figure 3.1A-C and E), this is consistent with the suggestion that while CALR is predominantly ER localized there were some factors bound to some cells, and the relevance of these remains to be investigated.

3.2.3 The CALR antibody gives a strong well defined signal in Western blotting in neuroblast cell lines.

Experiments from the Ochiai group have been performed using CALR on lysates taken from sperm collected from the epididymis and see below of note (Shaw, et al, 2013). It was further important to determine if the antibody would detect the presence of CALR when isolated against lysates taken from the human cell lines to be used. Initially only lysates were generated from H7100, OEC21 and OEC33 cell lines by the standard method (see Chapter 2.2.5) and equal amounts of protein per cell were run on a 10% SDS page gel, under reducing conditions before transfer to PVDF membrane. Membranes were blocked then probed with a 1:1000 dilution of the CALR antibody for 1 hour at room (Figure 3.3A), developed to see if there was any CALR signal and then re-probed with a 1:1000 dilution of an antibody against FOD (Figure 3.3B) as a loading control. From the experiment it could be seen that the antibody strongly and specifically detected the presence of CALR in all the cell lines tested (Figure 3.3A). There appeared to be slightly less expression of CALR in OEC21

cells had a re-profile of the transcriptome with the TCR antibody (Figure 3.20). Indeed the protein binding may not have been equal across all the lanes. After the initial set of experiments it was decided to include OE19 cells in the analysis. Liposomes generated from OE19 cells were, using the same method as used to generate liposomes from 4T10B4, 12O1 and 12O13 cells and then under the same conditions as the previous experiments. The membrane was probed with a 1:1000 dilution of the CALR antibody (as used reliably alongside a commercial antibody against E19.5 tail signaling G117) (Figure 3.22). CALR was detected in the OE19 tail line, although the signal was lower than was previously reported from the other cell lines tested (Figure 3.34). Eqs7^{fl} floxed mice gave a strong signal suggesting that the poor signal from CALR may have been due to an issue with transfer or detection.

3.3.4 The CALR antibody shows strong expression in the exocrine pancreas but not in the lumen of Langerhans.

Exocrine pancreatic tissue was incubated with a 1:200 dilution of the CALR antibody. CALR signal was detected in brown-stained cells (Figure 3.42). The overall staining was even across the tissue (Figure 3.44) with CALR protein expression present in the glandular cells of the exocrine pancreas but not the lumen of Langerhans. The signal appears to be consistent across all the areas of the tissue tested (Figure 3.4 (B-C)). This data compares favourably with data obtained from the human pancreas atlas, which shows medium intensity of CALR staining in the exocrine pancreas and no staining in the lumen of Langerhans in the majority of the tissues they tested (Chen, et al., 2017). In contrast, Chikara et al. showed an significant increase in liver CALR expression between lean and obese Zucker rats. They showed very low very low levels of CALR expression in lean mice which was significantly increased in obese mice in response to ER stress/UPR activation (Chikara, et al., 2015).

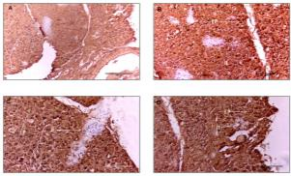


Figure 3. Distribution of CD44 in various tissues. Brown color shows the positive reaction of CD44. (A) Kidney, (B) Liver, (C) Spleen, (D) Testis. Magnification: (A) 400x, (B) 400x, (C) 400x, (D) 400x.

This experiment along with the previous Western blotting data and the immunofluorescence experiments suggest that the CALB-30-derived antibody is appropriate for Western blotting, immunofluorescence and in immunohistochemistry for the detection of human calcitonin in cell lines and tissues.

3.3.5. Validation of CANX expression using an antibody previously validated in human cell lines and tissues.

The anti-calcitonin antibody, also tested in detail and provided as a kind gift by the Chiba group was also tested. Initial immunofluorescence experiments were conducted with HT1080 cells using three antibody dilutions (1:20, 1:50 and 1:100) gave variable results (Figure 3.5), but did confirm that a 1:50 dilution of the CANX antibody (Figure 3.5B) was worth investigating further (Figure 3.5D). Going forward, it was decided to use a 1:50 dilution of the CANX antibody to test its expression in the untagged cell lines OEC1 (Figure 3.6E) and OEC3 (Figure 3.6F). Expression of CANX in OEC1 and OEC3 cells was comparable. In most cases with that of OEC (Figure 3.6C) and was comparable at 80 magnification. There appeared to be none of the possible cytoplasmic staining observed with the CALB antibody, which was as expected given that CANX is an ER membrane bound (Mishra, et al., 1997). The detection of the CANX antibody was likely to be masked using OEC10 cells (Figure 3.6 A-C) although the staining pattern was more difficult to see. In OEC10 cells due to their propensity to clump together.

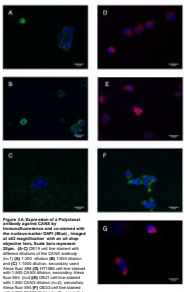


Figure 3. Fluorescence microscopy images of a *P. putida* cell.
 Fluorescence microscopy images of a *P. putida* cell expressing various fluorescent markers. The cell is shown in panels A through G. The markers are: (A) GFP, (B) mCherry, (C) mCherry, (D) mCherry, (E) mCherry, (F) mCherry, (G) mCherry. Scale bars are shown in the bottom right of each panel.

The experiment did confirm that, in OE19 cells, CANX appeared to be specifically associated with the EC2 and higher membrane and that a 1:500 dilution provided the best results. Although co-localizing experiments would be required to determine the extent of co-localization with other ER resident proteins.

3.3.6 The CANX antibody detects CANX by Western blotting in human neurosparged cell lines.

Like the CALR antibody, it was important to determine the behaviour of the CANX antibody in Western blotting of lysates of the same cell lines that were tested with the CALR antibody. Therefore, SDS-PAGE was carried out along the same lysates generated from the M7108, OE21 and OE23 cell lines that were used to examine the CALR antibody. Lysates were run on a 10% gel and proteins were transferred to PVDF membrane and blotted with a 1:1000 dilution of the CANX antibody (Figure 3.76). Additionally the CANX antibody was also tested using the OE19 cell line at the same time as the CALR antibody was tested (Figure 3.77). From the first experiment (Figure 3.76, and B) it can be seen that the CANX antibody produced a well defined signal in all three cell lines. Interestingly there appears to be a second band, represented in the OE23 cell lines that apparently appears in the M7108 and OE21 cell lines. This second band was also seen faintly in the OE19 cell line when the was tested (Figure 3.77). At first it was thought that this may be an experimental error caused by a slipping of the film during exposure to the ECL. However when taking into consideration the expression of Pdgfra and the signal band expressed by CALR (Figure 3.3C), which were loaded on the same SDS-gel gel, then it is possible that the observed band is a low abundance truncated form of

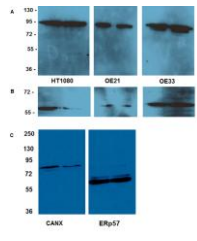


Figure 3-7 Detection of CAXX in Hepatocellular Carcinoma (HCC) Cell Lines. HT1080 and OE1, OE33 and OE21 cell lines were prepared and cultured in 10% FBS DMEM medium for 48 h. Cells were harvested and lysed in RNeasy lysis buffer. Total RNA was extracted using RNeasy spin columns and treated with DNase I. Total RNA (100 ng) was reverse transcribed into cDNA using the PrimeScript RT reagent kit. The cDNA was amplified using the CAXX and ERp57 primers. The PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide. The gel image was captured under UV light. The results are shown as representative images of three independent experiments. The data are presented as mean \pm SD. *p < 0.05, **p < 0.01, ***p < 0.001.

CANX expression in the OE19 and OE21 cell lines, which is not present in the OE21 cell line. The OE21 cell line was subjected to an equal number of passages in each passage, whereas both the OE19 and OE21 cell lines were originally derived from adenocarcinoma cells taken from the liver neoplasia (see Chapter 2.2.2) which may suggest that some form of gene translocation/modification event is CANX in the OE19 and OE21 cells that doesn't happen in the OE21 cells.

3.2.7. CANX shows strong expression in the human exocrine pancreas

In the same manner as the tissue sections were stained for CALR (Figure 3.4), human pancreatic tissue was incubated with a 1:200 dilution of the CANX antibody. DAB stained and then co-stained with hematoxylin (Figure 3.5). Overall the staining was seen across the tissue, indicating high expression of CANX particularly in the exocrine pancreas (Figure 3.5A). There was weaker expression in the blood vessels (Figure 3.5B), as indicated by lighter staining and only some of the pancreatic ducts appeared to be expressing CANX (Figure 3.5B compared to Figure 3.5C). The lack of argenteum appeared not to be expressing CANX (Figure 3.5D), which is in conflict with the data generated by the human protein atlas which suggests that, with the NP400A13 (Figure 1P03036 (Figure) and si-4016 (Gene Ontology) antibodies, and CANX expression in the tissue appeared to be medium to high (Dolan, et al., 2017). Comparison with other CANX antibodies would be required to resolve this issue, but was not within the scope of this thesis.

In summary the both the CALR and CANX antibodies performed well across a range of methods and with various cell lines and tissues, validating the methodology

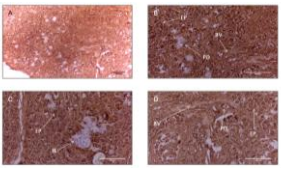


Figure 11. Histological and IHC of Human Pancreatic Ductal Adenocarcinoma. Hematoxylin and eosin (H&E) stained sections. The images of Figure 11A-D show the tumor cells (H&E stained) and the surrounding normal pancreatic acinar cells (H&E stained). The images of Figure 11E-H show the immunohistochemical staining for p53 (H&E stained) in the tumor cells (H&E stained).

**Investigation of the
Expression Profile
of GPx7**

4.1 Introduction

As we briefly discussed in Chapter 1, there is a possible link between dysfunction of ER resident chaperones and the resolution of the normal equilibrium established of the endoplasmic reticulum (ER) and ultimately, in some cases, to OAC.

4.1.1 The Glucosylase Peralataase family in Barrett's Oesophagus

Members of the GPV family of proteins have been observed to have a role in cellular protein folding, disulphide bond formation and the response to stress in the ER (Chapter 1.1). The glucosylase peralataase, GPV, has been shown to have a role in preventing the leakage of ERD14 generated H₂O₂ from the ER into the cytosol in HEC293 cells (Barnes, et al., 2014). It prevents SO loss of GPV by hypermethylation of its promoter region (Lee, et al., 2005) gene has an association of methylation (M) which may have the development of OAC. Additionally, there is evidence that GPV may be elevated by location specific methylation in OAC (Peng, et al., 2014). Furthermore GPV has been implicated in the control of downstream elements of the NF- κ B inflammatory pathway such that overexpression of GPV may result in the activation of NF- κ B signaling via TNF- α (Peng, et al., 2014). There is strong evidence that TNF- α and other NF- κ B mediated proinflammatory cytokines, such as IL-6 and IL-1 β are elevated in OAC and OAC (Thappa, et al., 2002) (Chattin, et al., 2005).

Finally overexpression of GPV promoted proinflammatory cytokines such as IL-6, IL-8, IL-1 β and oxidative stress. In the esophageal cell lines SMD-T and CP-A, and reduced oxidative DNA damage and double strand breaks (Peng, et al., 2015). Further, expression of GPV may have a role as a tumor suppressor. Peng et al showed that the knockdown of GPV expression in HET1A, an immortalized normal

oesophageal cell line resulted in an increase in growth, a decrease in the tumour suppressor p21, p27, p16 and p19 and an increased degradation of retinoblastoma protein (Rb) by phosphorylation. Conversely the expression of GP27 in OEC20 cells resulted in reduced phosphorylated Rb and an increase in p21, p27, p16 and p19 (Feng et al., 2016). This evidence suggests that an increased expression of the protein of the GP27 family may play a role in the pathogenesis of Barrett's Oesophagus and in the transition from stable BO to OAC.

4.2 Expression of GP27 in immunoblast cell lines

4.2.1 Immunofluorescence studies of GP27

Feng et al claimed that GP27 was down regulated in BO and OAC (Feng, et al., 2016). In order to study GP27 a number of experiments were undertaken. Initially, a panel of immunoblast cell lines were screened for GP27, with PDX as a control, in order to determine if GP27 was expressed and whether that expression was localized to the ER (Figure 4.1). All the cell lines used were immunoblast cancer cell lines derived from different cancer types: HT1080 from a fibrosarcoma, Hepa from human cancer, OEC2 from a squamous cell carcinoma of the upper aerodigestive tract, OEC30 cells from an adenocarcinoma of the lower aerodigestive tract and finally OEC19 cells, that were derived from an adenocarcinoma of the gastric cardia/oesophageal gastric junction (see Chapter 2, section 2.2).

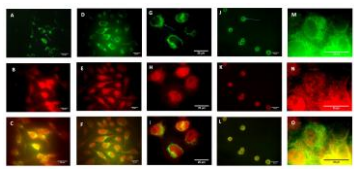


Figure 4-1 Confocal images of GFP-2 in the transmembrane domain. Top row: GFP-2 localization in the transmembrane domain of the plasma membrane. Middle row: GFP-2 localization in the transmembrane domain of the Golgi apparatus. Bottom row: GFP-2 localization in the transmembrane domain of the endoplasmic reticulum. Scale bars: 100 nm.

Each cell line was cultured to 70-80% confluence then treated with a polyclonal antibody against GFP1 with Abcam Ab9 (anti-GFP1) and a commercial monoclonal GFP2 antibody with Abcam Ab9 (anti-GFP2). Figure 4.1 shows the results for each cell line showing the 488nm excitation channel, the 647 excitation channel and a composite image. All the cell lines were positive for GFP1 expression (Figure 4.1A, C, G, J, M) and that expression appeared localised to the ER, with the exception of the HeLa cell line (Figure 4.1O) where the expression appeared predominantly localised to the ER but which also demonstrated a small degree of cytoplasmic staining. In contrast the expression of GFP2 appeared to be nuclear in all the cell lines, with the most apparent in the appropriate composite images for each cell line (Figure 4.1C, F, I, L, O). From the series of experiments it appeared that GFP2 was expressed in all cell lines, although it did not appear to be localised specifically to the ER. This could mean the possibility that the GFP2 antibody could be detecting a non-specific protein or other members of the GFP family.

4.2 GFP2 could not be detected in cell lines by Western Blotting

In order to determine if the antibody was binding specifically to GFP2 cell lysates from each cell line were analysed by 10% SDS PAGE and then blotted for GFP2. Blots using H11086, HeLa, Q21 and Q22 cell lines are presented (Figure 4.2A). According to the manufacturer's product data sheet, for the mouse monoclonal antibody (PT66) ab3727 (Jackson, 2017) an ~26kDa band was expected. Despite several attempts there was no detectable band in the H11086, HeLa and Q21 lanes. However, in the Q22 lane several bands were visible in ~35kDa and ~38kDa molecular weights (Figure 4.2A).

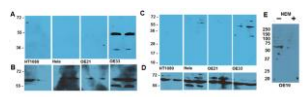


Figure 1. Western Blot of Overexpression of GAD65 in HEK293T Cells with a 1:1000 GAD65 Antibody. HEK293T cells were transfected with various GAD65 constructs (WT, Δ1-100, Δ101-200, Δ201-300, Δ301-400, Δ401-500, Δ501-600, Δ601-700, Δ701-800, Δ801-900, Δ901-1000) and analyzed by Western blotting with a 1:1000 GAD65 antibody. The results are shown in Figure 1. The results show that the 1:1000 GAD65 antibody is specific for GAD65 protein.

By using negative GP27 region immunoblot with anti-PC10 antibody that covers the protein was successful (Figure 4.2B). In order to control for possible loss of GP27 protein, the experiment was repeated using a GFP tag (Figure 4.2C). Again there were no visible bands at the 25kDa molecular weight marker. However the same 25kDa band is visible in the CE23 lanes as well in the 10% gel experiment (compare Figure 4.2A, panel 4, with Figure 4.2C, panel 6). Finally an experiment was conducted using CE19 (Figure 4.2C) that had been tested with and without 250nM NEM. This experiment was performed using a GP27 protein immunoblotting protocol by the Wang Group (see Chapter 2.2.1) (Wang et al., 2014). Without NEM there was a clear strong band between the 25kDa and 75kDa molecular markers and faint signals at higher molecular weights. In the presence of NEM there was a decrease in the signal compared to the sample without NEM, in addition to the faint bands seen at higher molecular weights. In both blue and nitro blue NEM samples there appeared a very slight spot at ~25kDa but it is not apparent if this is a true signal or not.

From the Western blotting data it appeared that GP27 was not detectable in the cell lines tested, despite the strong antibody giving a strong nuclear staining pattern by immunofluorescence. This appears to contrast with the published literature from Peng et al. which showed that, in CE23 cells, GP27 expression was upregulated by site specific promoter methylation at the transcriptional level (Peng et al., 2014). The appearance of bands at other molecular weights and the data seen from immunofluorescence (see section 4.2.1 and Figure 4.1) strongly suggests that, in the absence of GP27, non-specific binding is occurring. Interestingly, more data from the human proteome atlas suggests that GP27 may have a nuclear localisation, from experiments using an independent antibody (see NP1462822) (Johari, et al., 2017).

In order to determine if the observed signal from the Hela and CHO cells just above in the CE19 cell line was non-specific binding, it was decided to transfect Hela cells with a GFP-V encoding cDNA with the goal of analyzing GFP-V expressing cells for their response to ER and bile acid stress. Hela cells were transfected (for protocol, see Chapter 2.2.4) with a construct that tagged GFP-V cDNA and along with an ESO-1α cDNA mutant (which had previously been successfully transfected into Hela cells) as a positive control. The cells were cultured to 80% confluence, in 6-well plates containing 3mm glass coverslips before transfection with 1μg/plate (2000 cells/plate) of the appropriate cDNA construct. The transfectants were then removed, fixed and analyzed by immunofluorescence (Figure 4.3). Non-transfected Hela cells were also imaged for comparison (Figure 4.3D). In the non-transfected cells the distribution of staining appeared non-specific, with background distributed throughout the cytoplasm. The staining in the transfected cells showed a higher distribution, however it didn't appear strongly localized to the ER (Figure 4.3A). Staining of the mutant (Figure 4.3B) showed that the cells had been transfected successfully as the presence of the tag was co-localized with that of the enhanced GFP staining (Figure 4.3C). By comparison the Hela cells transfected with ESO-1α show staining which appears more ER based (Figure 4.3E) when compared to the staining observed in the non-transfected Hela cells (Figure 4.3A). This is equivalent to the staining observed (Figure 4.3D) when these appeared to be myc staining in those where there was no ESO-1α expression.

Overall it was difficult to detect GFP-V in transfected Hela cells.

2.2.2 GP27 is expressed in Treg cells but was not detectable in human pancreas, B-cell's neoplasm or aneuploidal gastric tumour.

In an attempt to see if GP27 was increased in B0s and in aneuploidal cancer human tissue was stained to detect GP27. It has been suggested that GP27 expression, may be high in the macrophage system (Ehnen, et al., 2017) (Pruett, et al., 2011) and in tissue obtained from oral smears was increased and detected for GP27. In addition human pancreatic tissue was stained as a negative control alongside the human aneuploidal tissue and the no ticks on the human protein atlas showed that pancreatic expression was low to none (Figure 4A) (Ehnen, et al., 2017).

Pancreatic tissue was stained with CD45 and haematoxylin only as a negative control (Figure 4A), to measure the positive control (Figure 4B) and for GP27 using the anti-GP27 antibody obtained from the Yang group (Figure 2.2.1) (Figure 4C). As expected from the literature (Ehnen, et al., 2017) GP27 staining was practically non-existent throughout the pancreatic tissue with a few areas of weak brown staining which were likely to be CD45 overexpression. The no ticks (Figure 4C) showed GP27 expression in both the Treg cells and in the neutrophil ducts which also agrees with the literature (Pruett, et al., 2011). In the tissue sample of B0 there appeared to be no expression of GP27 in the epithelial squamous epithelium (Figure 4C) although there was an indication of slight expression in the lamina propria. Other than expression in lymphocytes, which have a high expression of GP27 (Ehnen, et al., 2017), there was a high indication of GP27 expression in the tumour tissue when from the aneuploidal gastric junction. However GP27 did not appear to be localized to the tumour cells and may have been in the lamina propria although the wasn't clear given the disorder of the tissue. This appears to correlate with the

cellular lines where GP2 expression was not detectable in uncoated cell lines, when analyzed by Western blotting.

Taken together there did not appear to be any expression of GP2 in tissues obtained from immortalized cancer cell lines. Similarly there did not appear to be any expression of GP2 in tissue taken from patient samples of BCL. However, there was no indication of expression of GP2 in the control tissues which suggests that the antibody obtained from the Yang group could be suitable for visualizing GP2 expression in tissue under the right conditions. (Yang, et al., 2014).

**Investigating the
Expression of
Prdx4 in the
Oesophagus**

5.1 Introduction

Prd1 is an ER resident scavenger of H₂O₂ (Gardai & Fisher, 2008), which, as discussed in Chapter 1, can allow H₂O₂ to act in a disulfide bond formation (Zawadzki, et al., 2008). Prd1, also, in the ER, is a dimeric form comprising of two Prd1 dimers (Figure 5.1). Each Prd1 polypeptide contains a single peroxidase catalytic site which can react with peroxide to become superoxidized. The superoxidized catalytic site then reacts with the resulting glutathione on the adjacent Prd1 polypeptide in Prd1 dimer to form a disulfide bond, which is then available to be oxidized to a reduced PDI family member (Stallard & Egeland, 2011). It has been suggested that, under normal conditions, members of the CYP family are responsible for the generation of H₂O₂ accumulation from oxidative protein killing within the ER and that Prd1 assisted detoxification only becomes applicable when levels of H₂O₂ in the ER are increased (Borrmann, et al., 2016). As discussed in Chapter 4 in BOD and CAC, there is evidence to suggest that the H₂O₂ scavenging capacity of the CYPs, such as CYP2, is impaired and so it then becomes important to examine how the activity and regulation of Prd1 is affected.

5.2 Prd1 expression in esophageal cell lines

Initial experiments to isolate antibodies and confirm the expression of Prd1 were conducted in the HT1080 cell line using immunofluorescence. HT1080 cells were cultured to approximately 70% confluence in 15cm plates (see also, 5.1.1) and stained with either Prd1 or PDI as a positive control (Figure 5.2). Comparing the cells stained with PDI (Figure 5.2A) with those stained for Prd1 (Figure 5.2B), Prd1 appeared to be expressed and localized to the ER. Non-specific stain (control HT1080 cells and mouse kidney, seen not on a 10% SDS-PAGE gel for

analysis of P2004 experimentally (Western blotting) (Figure 1.2C), with P201 expression as a positive control (Figure 1.2C, lanes 5 and 6). Finally, no signal could be detected for P201 in the lanes stained by P201 antibody that transfer had been successful. Staining of the membranes with peroxidase 5 (P (Figure 1.2D), confirmed that the transfer had been successful, although some degradation had occurred in the Protein Lanes (lane probably due to the freezing or thawing process).

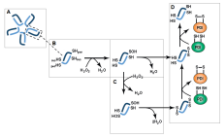


Figure 15 The Electron Transport Chain Proton motive force is a measure of the energy stored in a proton gradient across a membrane. The electron transport chain uses the energy from the oxidation of NADH and succinate to pump protons across the membrane. The electron transport chain consists of several complexes (I, II, III, IV) and mobile electron carriers (Q, C). The electron transport chain is located in the inner mitochondrial membrane. The electron transport chain is a series of redox reactions that transfer electrons from NADH and succinate to oxygen, which is reduced to water. The electron transport chain is a key component of cellular respiration and is essential for the production of ATP.

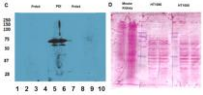
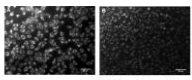


Figure 2 Effect of compounds 1, 2, and 3 on the proliferation of HT29 cells. **(A)** Micrographs of HT29 cells treated with 10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M of compound 1. **(B)** Micrographs of HT29 cells treated with 10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M of compound 2. **(C)** Micrographs of HT29 cells treated with 10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M of compound 3. **(D)** Western blot analysis of HT29 cells treated with 10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M of compound 1, 2, and 3. **(E)** Western blot analysis of HT29 cells treated with 10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M of compound 1, 2, and 3. **(F)** Western blot analysis of HT29 cells treated with 10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M of compound 1, 2, and 3. **(G)** Western blot analysis of HT29 cells treated with 10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M of compound 1, 2, and 3. **(H)** Western blot analysis of HT29 cells treated with 10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M of compound 1, 2, and 3. **(I)** Western blot analysis of HT29 cells treated with 10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M of compound 1, 2, and 3. **(J)** Western blot analysis of HT29 cells treated with 10⁻⁶ M, 10⁻⁵ M, and 10⁻⁴ M of compound 1, 2, and 3.

In order to determine if PtdM was expressed in nonmyelinating cell lines, immunofluorescence was performed on OE19 and OE33 cells (Figure 5.1). OE19 cells were stained with PDI (Figure 5.1A) and PtdM (Figure 5.1B). Comparing the OE19 cells stained with PtdM with those stained with PDI as a positive marker of cell staining, PtdM appeared to be localized specifically to the ER. Comparing PtdM expression in OE33 cells (Figure 5.1C) with OE33 cells stained for PDI (Figure 5.1D) again the expression of PtdM appeared to be localized specifically to the ER. This was similar to the result obtained in H7700 cells (Figure 5.2). An OE19 cell lysate was run on a 10% polyacrylamide gel under reducing conditions, with and without the presence of NEM to tag disulfide bonds. PtdM was detected at the expected 25 kDa band (Figure 5.1E) and in addition to this a faint band was detected around the 50 kDa marker in the OE19 lysate with NEM. In order to determine if this was a non-specific result, OE19 and OE33 cell lysates were run with and without NEM under reducing conditions (Figure 5.1F) and non-reducing conditions (Figure 5.1G). It was found that PtdM was detected at the expected molecular mass of ~25 kDa in the OE19 and OE33 cell lysates, under reducing conditions. In addition, OE19 cells showed a faint band at ~50 kDa and a faint band was detected at around 75 kDa (Figure 5.1F). In contrast, the 50 kDa and 25 kDa bands were absent from OE33 lysates.

When reduced by the interaction with NEM, PtdM co-localized to PDI in and disulfide bond formation within PDI (Chen et al., 2008). It is therefore possible that in the reduced samples, the NEM has tagged the interaction between PDI and PtdM (Figure 5.2F).

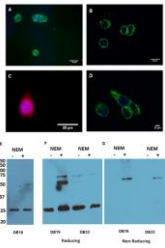


Figure 2 Localization of DAP2 in HEK293T cells. HEK293T cells were transfected with DAP2 and HA. (A) HEK293T cells transfected with DAP2 and HA. (B) HEK293T cells transfected with HA. (C) HEK293T cells transfected with DAP2 and HA. (D) HEK293T cells transfected with HA. (E) Western blot analysis of DAP2 protein levels in HEK293T cells transfected with DAP2 and HA. The blot shows bands for DAP2 in lanes 1, 2, and 3, and a control lane 4.

3.3 Expression of P/CAF in human tissue

With the observed expression of P/CAF in the oesophageal cell line models of BC and OAC, it was important to assess expression in other relevant human tissue samples of BC and OAC. Compared to samples taken from normal oesophageal and gastric tissues. Samples of normal oesophageal, normal gastric, BC tissue and tissue taken from an oesophageal gastric junction tumour were therefore immunohistochemically stained for P/CAF. In addition, immunohistochemical expression of P/CAF in High (Zhan, et al., 2017), human pancreatic tissue was also studied as a positive control (Figure 5.4). As expected in the pancreatic, expression of P/CAF was high in the exocrine pancreas and low to none in the islets of Langerhans tissue (Figure 5.4). P/CAF was expressed in the normal oesophageal tissue (Figure 5.4C) compared to control (Figure 5.4 B). No clear expression appeared but when comparing it to the P/CAF expression in normal gastric tissue (Figure 5.4D). In the present samples of BC tissue, there was a discrepancy in the level of staining between those areas which had the appearance of squamous epithelium and those which contained cells with the morphology of gastric colunar cells (Figure 5.4E and F). This appeared consistent between different samples, allowing for between patient variation.

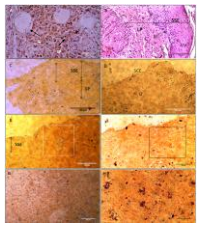


Figure 1. Histological images of the tissue. (A) Low-magnification image of the tissue. (B) High-magnification image of the tissue. (C) Low-magnification image of the tissue. (D) High-magnification image of the tissue. (E) Low-magnification image of the tissue. (F) High-magnification image of the tissue. (G) Low-magnification image of the tissue. (H) High-magnification image of the tissue.

In the future, we could obtain more patients with *miR-101-3p* expression in colon cancer (Figure 5.42 and 5.43) to further confirm the pattern of staining observed. There was evidence that some areas may have a darker staining, which may indicate localized areas of increased *PRDM14* expression, however this wasn't clear in one of the patient samples (Figure 5.42). In another sample, however, there were clearly identifiable areas of staining, possibly indicating areas with high expression of *PRDM14* (Figure 5.44). This correlates with the observations that *PRDM14* expression in breast and prostate cancers is increased in areas of carcinoma compared to non-tumour tissue (Wang, et al., 2010). Similar increases in *PRDM14* expression have also been found in Oral Cavity Squamous Cell Carcinoma and in Glioblastoma multiforme, an aggressive brain malignancy (Cheng, et al., 2011) (Kim, et al., 2012).

Taken together, the data suggest that *PRDM14* expression is worth pursuing as a possible marker of GI cancer in a larger scale study.

Investigating AGR2

a Novel PDI family

Member

-

6.1 Introduction

Protein Cdk20 (AGG2) is a member of the CDK family. Over expression of AGG2 has been shown to increase the rate of cell division, as discussed in Chapter 1 (Chapter 1.14), reviewed in (Bryant, et al., 2011). Research into AGG2 has suggested that it may also have a role in BC and its transformation into DAC through the silencing of the tumor suppressor p53 (Pines et al., 2006).

AGG2 is involved in the production of steroid esters (Wu, et al., 2002) and has been linked to the response to stress in the ER (Zhang, et al., 2007) (Chen, et al., 2010). Research has shown that AGG2 can interact with BIP/GP78 and that this interaction is dependent on AGG2 dimerization (Wu, et al., 2012). However, the exact role that AGG2 has in the ER stress response is unclear. There is also evidence to suggest that it may also form complexes with other ER resident proteins either as a monomer or as an interaction between a target protein and its chaperone (Chen, et al., 2010).

6.2 AGG2 Expression in Human Tissue Sections

Tissue blocks of normal esophageal, normal gastric, BC and DAC from human patients, were included, together with paraffin-embedded tissue and then stained for AGG2 (Figure 6.1). AGG2 was present in normal gastric tissue, in the columnar glandular cells (Figure 6.1B) but was not present in normal esophageal tissue (Figure 6.1A). The tissue sections taken from the esophageal glandular tissue, stained with BC (Figure 6.1C and 6.1D) show a strong reactivity with the presence of gastric columnar cells (Figure 6.1C) and glandular tissue (Figure 6.1D). AGG2 expression appeared high in the columnar glandular cells (Figure 6.1C) and in the glandular tissue (6.1D) while remaining low, in comparison, in the normal esophageal

epithelium (Figure 6.4C and 6.5). In the tissue sections taken from patients diagnosed with OAC (Figure 6.1E and 6.7), AGS2 expression appears confined to the granular cells (and hence there is some suggestion of AGS2 expression in the undifferentiated cellular region of one of the samples (Figure 6.7F)).

From the experiment 1 appears that AGS2 is expressed in the columnar epithelium of gastric tissue but not normally expressed in the endoplegma, consistent with the literature. In comparison AGS2, in DC, is only expressed when the squamous epithelium has transitioned into glands or columnar epithelium. This is probably due to AGS2's involvement in the production of mucin. In the samples of OAC, AGS2 is expressed mostly in the columnar cells but there is some suggestion that AGS2 may also be expressed in the tumour stroma.

6.3 Expression of AGS2 in Oesophageal Cell Lines

In order to determine if AGS2 was also expressed in oesophageal cell lines, OE19 and OE33 cells were cultured to approximately 70% confluence on 15mm glass cover slips. The cells were then fixed with 4% paraformaldehyde and then permeabilised in ice cold methanol before blocking for an hour and then incubated overnight at 4°C with either an anti-AGS2 antibody or PC1 antibody as a positive control of ER localisation. Each cover slip was then incubated with an appropriate fluorescent secondary antibody and/or stained with DAPI (Figure 6.3). Comparing OE19 cells stained with AGS2 (Figure 6.2E) to OE19 cells stained with anti-PC1 (Figure 6.2A) showed that, in OE19 cells, AGS2 is localised to the ER. In comparison the expression of AGS2 in OE33 cells was only present in a few cells (Figure 6.2C), and these cells which showed some expression did not present with

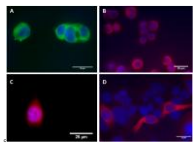


Figure 3 Expression of NRP1 in embryonal cell lines. H1hESC (A) and H1ESC (B) cell lines were stained with anti-NRP1 (green) and DAPI (blue) antibodies. H1ESC (C) and H1hESC (D) cell lines were stained with anti-NRP1 (green) and DAPI (blue) antibodies. Scale bars represent 10 μm.

Comparing the expression of AGS2 between the OE-19 and OE23 cell lines showed that the expression of AGS2 was strongly repressed in the OE-19 cell line (Figure 6.2B) compared to OE23 cells (Figure 6.2C). In the OE-19 cell line, AGS2 was expressed in each cell whereas in the OE23 cell line only a very small amount of AGS2 was expressed in each cell.

To investigate the further cell types of OE-19 and OE23 cells were investigated by SDS-PAGE under reducing conditions and Western blotted with an anti-AGS2 antibody (Figure 6.3A). The OE-19 cell lines showed a good signal at the expected molecular weight which was absent in the OE23 lanes. Based on this result, the SDS-PAGE was repeated using OE19 cell lines under both reducing and non-reducing conditions. To explore whether AGS2 formed any complexes with other proteins OE19 cell lines were run with or without NEM to preserve disulfide bonds (Figure 6.3B). Under reducing conditions, without NEM, the result of the experiment was similar that seen in Figure 6.3B lanes 1 and 2. Under non-reducing conditions, without NEM, only the expected bands appeared (Figure 6.3B lanes 3 and 6). However in the samples with NEM bands were visible at 27 kDa, 55 kDa and around the 75 kDa molecular marker (Figure 6.3B lanes 7 and 8). In the samples run under non-reducing conditions, with NEM, the band seen at 27 kDa could possibly be AGS2 in a dimeric form, as seen by Raju et al and so there is a possibility that another of the bands may be an association with BIP (Raju et al., 2013).

absence of the presence of these bands, therefore primarily even suggests that AGSD can form multiple complexes with the CE-mediated complex.

In order to determine if the observed AGSD interaction were truly dependent on CE-19 cells were cultured in confidence and then treated with reducing reagents. To establish reducing conditions, CE-19 cells were cultured in three EGM dishes, one with each used as a control, one was treated with 5mM DTT and one dish was first treated with 5mM DTT for 5 minutes then washed and incubated in media for a further 10 minutes. This was done to see if removal of the reducing conditions resulted in a return to the control state, which would indicate that the complex formation was reduction-dependent. Similarly the experiment was repeated under oxidizing conditions with 5mM Dithiothreitol to determine if the complex formation was oxidation dependent. After each treatment, including the control, samples were washed with 20mM HEPES for 5 minutes, to preserve any disulfide bonds then fixed and stained for AGSD under non-reducing conditions with HRP (Figure 6.4). In the Western blot of the samples under reducing conditions (Figure 6.4L), the control samples exhibited a similar pattern of bands as previously seen (Figure 6.3E). These bands disappeared when the CE-19 cells were treated with 5mM DTT leaving only the main AGSD monomer band. When CE-19 cells were treated with 5mM DTT and then allowed to recover by incubation for a further 10 minutes with untreated media there was a noticeable return of some of the extra bands. The disappearance and their recovery strongly suggests that the interaction disulfide are truly dependent.

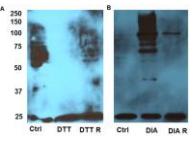


Figure 4 HSP70 and HSP90 are induced in cells treated with DTT. **Panel A:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min. **Panel B:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH. **Panel C:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel D:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel E:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel F:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel G:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel H:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel I:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel J:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel K:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel L:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel M:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel N:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel O:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel P:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel Q:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel R:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel S:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel T:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel U:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel V:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel W:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel X:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel Y:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT. **Panel Z:** HSP70 levels were analyzed in cells treated with DTT (100 μM) for 15 min in the presence of 100 nM CSH and 100 nM DTT.

The experiment was then repeated with Chondria as an additional agent (Figure 6.46). Examination of the Western blot showed similar control patterns of bands. However they were higher than seen in the previous experiment. The addition of Small Chemicals to OE 19 cells clearly showed an increase in the number and signal strength of the extra bands. Adding the OE 19 cells to medium for 10 minutes after Chondria treatment showed a return to the band pattern seen in the control, with the exception of a strong band at 100 kDa (Figure 6.46). This suggests that Chondria increases the formation of extra bands.

These experiments demonstrate that the observed extra bands are index dependent and that these extra bands represent a AGS2 complex formation. This suggests that the ability of AGS2 to form complexes with other proteins is index dependent and suggests a role for AGS2 in cell cycle protein folding despite the lack of a conventional CDC2 motif in the α domain.

POI is involved in cell cycle protein folding pathways in the ER. It was therefore expected that it was one of the main proteins that complexed with AGS2. To investigate this an immunoprecipitation was prepared. Protein A sepharose beads were incubated with anti-POI antibody before immunoprecipitating POI from OE 19 cell lysate. The bead-bound mixture was centrifuged, washed and the beads collected in sample buffer as detailed in Chapter 2.3.7. The immunoprecipitate was then analysed by SDS-PAGE on a 12% polyacrylamide gel under reducing conditions, without NEM, alongside a mixture of lysate, and POI antibody (PK3-D16, Abcam) and beads and input OE 19 lysate (Figure 6.45) prior to Western blotting with an anti-AGS2 antibody.

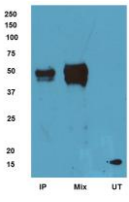


Figure 3.3 Immunoprecipitation of GPR124 and Synapin
 GPR124 was immunoprecipitated with Synapin (100 ng/ml) from HEK293T cells transfected with GPR124 and Synapin. The immunoprecipitates (IP) and the mixture of GPR124 and Synapin (Mix) were analyzed by Western blotting. The immunoprecipitates were analyzed by Western blotting. The immunoprecipitates were analyzed by Western blotting. The immunoprecipitates were analyzed by Western blotting.

AGS2 was observed in the untreated form as expected but was not present in the form of P (Figure 4C) suggesting that treatment of the experiment. AGS2 and AGS2 do not form detectable complexes in the ER.

In summary AGS2 was found to be expressed in normal gastric tissue but not in the normal oesophagus. AGS2 expression in patient samples of BC was found to be consistent across the four subtypes. The treatment of the gastric tissue (CNC, AGS2 expression was found both in the ER and closely associated with the luminal area which could suggest that, in CNC, the tumour produces and secretes AGS2. Examining AGS2 expression in oesophageal cell lines, AGS2 was detected in OE19 cells but not in OE23 cells, when analysed by Western blotting. In oesophageal cancer, AGS2 was found to be expressed strongly in the ER of OE19 cells. In OE23 cells, AGS2 expression was only seen in a few cells and did not appear to show classical ER expression. This suggests that AGS2 expression may occur in OE23 cells but it is not clear whether this is due to differences in AGS2 expression or to differences in the way that AGS2 is processed. Further analysis of OE19 cells under reducing conditions, in the presence of NEM. Further analysis of OE19 cells under reducing conditions showed that the high molecular weight oligomers were more abundant, suggesting that AGS2 may form a stable trimeric protein and perhaps constitute protein folding in the ER of OE19 cells.

Discussion

-

2.1 Introduction

This study was based on the hypothesis that ER stress leads to the dysregulation of ER membrane-binding molecules in the endoplasmic and that this ER dysregulation may play an important part in the disease BD and its transition to GAC.

Initial experiments focused on the proteins GRP78 and PDI which play a role in both protein folding, via the ERCH1-PDI pathway and in protecting the ER from ROS, a by product of ERCH1-PDI protein folding (Nguyen et al., 2011) (Chen et al., 2014). This was followed by the identification of AGRP2, a member of the PDI family of proteins, which, due to its possible involvement in various cancers (Brython, et al., 2011), became a promising candidate for research into the transition of the amygdala to BD and its acute precursor GAC.

Experimentally, some improvements could be made in the way the study was carried out to improve the weight of the results. The Western blotting experiments would benefit from more consistency, such that each blot should have followed the same format of radioligand-reducing and +NEM. In addition each blot should have included PDI as a positive control and been repeated with each.

Similarly the controls for the immunobiochemistry experiments could be improved. Ideally pancreatic tissue was stained against insulin with an anti-insulin as a positive control. This should have been continued forward with all subsequent experiments. In relation to the AGRP study, if the study was repeated, it would benefit from the inclusion of a negative control of a ligand not known to bind with overdevelopment. Suitable negative controls would be human tissue taken from the brain, muscle, adipose or skin which have low expression scores of AGRP, according to the Human Protein Atlas (Zhang, et al., 2017).

3.2 The Expression of GP2 in the ER, SO and DAC

GP2 has been shown as a component of FOL2 in the ER and can collect FOL2 in a disulfide-linked binding to connect with SEC23 and FOL1 (Figures 4.1 and 4.2) (S. Valencia from literature suggested that GP2 may play a role in the pathogenesis of SO and DAC). Experiments with GP2 had showed that it had a protective role in preventing oxidized DNA damage in colonocytes (Sheng, et al., 2011) and that it had tumour suppressor capabilities which were down regulated in cancer cells by location specific methylation of the GP2 promoter region (Peng, et al., 2016). Immunofluorescence of the cell lines HT1080, CE18, CE21 and CE33 showed some expression of GP2 but the expression did not appear to be specifically localizing to the ER when compared to FOL2. In this thesis Western blotting of cell lines taken from HT1080, CE18, CE21, CE33 and HeLa cells failed to show any expression of GP2 at the expected molecular weight in the manufacturer's data sheet. A heavy non-specific signal was seen in some experiments, for example in an CE33 cell line, but this appears as a higher molecular weight than expected (Figure 4.2). The GP2 antibody reactivity towards CE18 cell lines were similar to the CE33 cell lines, in that multiple bands could be seen at higher molecular weights but not at the expected molecular weight for GP2. Considering the experimental data from both Western blotting and immunofluorescence together they seem to suggest that there was some non-specific binding with the antibody and a lack of GP2 expression. The lack of an expression compared with other molecules that GP2 is expressed in cancer derived cell lines as described in the literature. Further experiments to determine the effects of ER stress on GP2 expression would either require a transfection approach, the verification of GP2 specific antibody, under a screen of cell lines to identify a cell type with high GP2 levels which have

been suggested to express CP2⁺. For example, Peng, et al did show baseline expression of CP2⁺ in Human Bladder of Blah T, HET-A, HSC2 and CP-A cells (Peng, et al, 2011) supplementary data, figure 1)

Human tissue samples of BC and OAC were kindly provided by James Cook University Hospital and explored for CP2⁺ to determine if there was any correlation between tissue expression of CP2⁺ and the results seen in the cell line experiments. In the tissue samples of BC⁺ expressed the CP2⁺ may be expressed in the lumen propria but there was no expression observed in the epithelial layer. In tissue taken from patients with OAC there appeared to be a small amount of a granular which was located close but did not appear to be associated with tumour cells. Given the disordered nature of the tissue, expression could equally be from another cell type, such as gastric like colunar cells rather than a tumour cell. In situ hybridisation or another complementary approach would be required to confirm CP2⁺ expression in the tissue.

CP2⁺ is homologous to CP2⁺ and merits further examination. In the context of this study, CP2⁺ is thought to perform a similar role to CP2⁺ in the ER, except that CP2⁺ is soluble in the ER lumen and CP2⁺ is membrane bound and localised to the lumen of the ER membrane (Chen, et al, 2010). CP2⁺ has also been shown to have a higher expression level than CP2⁺ (Nguyen, et al, 2011). In tissue, CP2⁺ is expressed in the digestive system. The Human Tissue Atlas shows that, possibly tissue and/or antibody dependent, expression of CP2⁺ in the esophagus and high expression in the stomach (Chen, et al, 2011). Therefore it may be possible compare any findings from the cell models to the disease.

It is also an essential factor to reduce production cost and transportation of the EEP system. Being operated from a single, centralized, location may also affect the H_2O_2 storage capacity of GPdH. As GPdH performs a role in preventing the leakage of H_2O_2 from the ER (Plambeck et al., 2014), then dysfunction of GPdH in ER could result in excessive reduced DNA damage (Figure 7-1) and ultimately cause the transition from stable Bax^{low} to CAC.



Figure 7-1. Under normal conditions GPdH prevents the leakage of H_2O_2 from the ER. The dysfunction of GPdH would allow the leakage of H_2O_2 which could result in excessive DNA damage and ultimately lead to a change from stable Bax^{low} to CAC.

3.3 Expression of PtdIn

Like GPV, PtdIn is an HSC oncogene in the ER. PtdIn can also act in cytoplasmic and transmembrane with GTP-binding proteins upon binding (Tremblay, et al., 2008). It is thought that GPV proteins use the main mechanism of the e case. H₂O₂ produced by oxidative protein folding in the ER and that PtdIn only folds that rate when levels of H₂O₂ are maximal (Dominguez, et al., 2014). As such the study of PtdIn becomes imperative to G₁ gain the evidence that GPV may be down regulated in BC (Pang, et al., 2016).

CE19 and CE23 cells were both shown to express PtdIn by immunofluorescence and Western blot (Figure 3.3). There were observable differences in the expression of PtdIn between CE23 and CE19 cells. In CE23 cells expression of PtdIn seemed less tightly regulated in the ER than in CE19 cells. Western blotting of CE23 and CE19 lysates under reducing conditions showed expression of PtdIn at the expected molecular weight and also showed bands at higher molecular weights, suggesting that there may be a difference in regulation between CE23 and CE19. The more advanced CE23 cells. PtdIn has been shown to form disulfenic complexes (Tremblay, et al., 2008), so under reducing conditions it is possible that the presence of NEMO may impact PtdIn in a number of different states. Under non-reducing conditions, in the presence of NEMO, PtdIn may be trapped as a disulfenic dimer. Interestingly there appeared to be a greater expression of PtdIn in CE19 cell lysates compared to CE23 cell lysates, although whether the expression differences are reproducible requires further experiments. Both cell lines were originally derived from primary OAC cultures, with the CE19 cell line derived from a tumour at the oesophago-gastric junction. While the CE23 cell line was originally derived from a tumour in the lower oesophagus with Barrett's metaplasia (Rickett, J., 1987). This

possibly suggest that cells located near the osteogenic genetic junction, which could be more likely to experience an increased exposure to cell, might more likely to express an increased PDL1.

In human tissue sections stained for PDL1, PDL1 appeared to be expressed higher in normal genetic tissue than in normal osteological tissue. In stained samples of 60 tissue, PDL1 expression did appear higher in more osteogenic genetic tissue compared to the areas containing epiphyseal apposition (Figure 1.4). This may possibly correlate with the difference observed between OC19 and OC23 cells, which suggests that PDL1 expression may be linked to the amount of genetic like cells in the area (19).

PDL1 expression in tissue taken from osteological genetic junction tumours differed between samples with some sections showing little to no expression of PDL1 and some sections showing localized areas of staining in or near tumour cells. PDL1 expression has been linked to a number of cancers. PDL1 expression has been observed to be increased in some of osteosarcoma, chondrosarcoma, osteocoma, compared to non-tumour tissue (Hsu, et al. 2006). Similar increases in PDL1 expression have also been found in Oral Cavity Squamous Cell Carcinoma and in Glioblastoma multiforme, an aggressive brain malignancy (Cheng, et al. 2011) (Wu, et al. 2012). Additionally, there is evidence that increased PDL1 may also be a factor in tumorigenesis. Increased PDL1 has been linked to cancer-induced osteoclastogenesis (Ruffin, et al. 2015) suggesting that overexpression of PDL1 may be a factor in the induction of OC in OC.

Immunohistochemical, both the experiments, in this thesis, suggest that normal osteological expression of PDL1 is low. It would be interesting to see if this is also

from OE19 and OE33 cells. It is then to be determined whether these cell lines express a 2. parvalbumin-like and/or parvalbumin-like OE19 and OE33 cells to determine if they give the same response and to see if less or more stress is needed to cause PMA expression in the above 3 cell lines. Expression analysis would be done on both cell lines and protein level. It may also be worth testing the needs of the cell lines, particularly under stress conditions to see if any PMA is secreted in response to stress conditions.

7.4 Expression of AGR2

AGR2 is a member of the PDZ protein family. It is localized to the ER by a C-terminal KDEL motif similar to the KDEL and KYKL ER retention sequences (Ding, et al., 2012). Like PDZ, AGR2 contains two PDZ domains which could interact with AGR2 in a stable protein binding. However, unlike PDZ, AGR2 contains a single active cysteine residue (Galligan & Peterson, 2012) suggesting that it is involved in disulfide bond formation. It may be via an intramolecular disulfide bridge to form a homodimer. It is shown that AGR2 can dimerize by forming a disulfide bond with itself (Clark, et al., 2016).

The experiment in Chapter 6, Figures 6.2 and 6.3, showed that AGR2 is expressed in both OE19 and OE33 cells. In OE19 cells AGR2 appears to be widely expressed in the ER but in OE33 cells there was a suggestion that AGR2 was not widely confined to the ER. There is a suggestion to suggest that AGR2 may not be widely able to be secreted (Foster, et al., 2016) so it is possible that in OE33 cells, some AGR2 is secreted. Comparing OE19 cells and OE33 cells, AGR2 appeared to be more highly expressed in the OE19 cell line than in OE33 cells. Western blot

analysis of lysates taken from OE19 and OE23 cells appear to support the view
AGSD was detected in OE19 lysates but not in OE23 cells in treated samples.

Further examination of AGSD expression in OE19 lysates showed clearly that, under
non-reducing conditions, AGSD can form a dimer. In addition detection of multiple
bands in non-reduced samples, in the presence of NEM, suggest that AGSD is also
capable of forming other proteins containing the dimer that AGSD may be involved
in the protein folding. Further experiments support this assertion, for example the
treatment of OE19 cells with DTT showed a reduction of all AGSD client interactions,
including the reduction of dimers to monomers. A DTT treatment and monomer
experiments showed that after recovery the AGSD dimer and other AGSD protein
interactions returned to the control value (Figure 5.4). Dimers cause an increase in
AGSD client interactions, which, when allowed to recover, returned towards baseline
expression. This evidence clearly indicates that AGSD client protein is more
dependent on the AGSD interactions are occurring through disulfide bond
formation.

Evidence in the literature suggests that AGSD interacts with BIP/Gp78 (Jin, et al.,
2013). Going forward it would be helpful to identify the AGSD client protein
partners using these experiments to try determine possible interactions before
conducting further co-immunoprecipitation experiments.

AGSD expression levels are also associated with several cancers, in particular
hormone dependent breast and prostate cancer and in hormone independent
colorectal, pancreatic and gastric cancers (Bryant et al., 2011). Due to AGSD's
association with cancer it was worth investigating if AGSD had any links to BCL2 and
C-MYC. Experiments with hormone independent and normal gastric tissue showed that

AGS2 was not expressed in the normal esophagus. AGS2 was found to be expressed at the levels in normal gastric tissue in the mucosa of the gastric cardia. AGS2 has a role in mucin production particularly the MUC2 mucin in the intestine (Egerton, et al., 2014) (Park, et al., 2005) and in MUC1 and MUC5AC (Frost, et al., 2012), so its presence in the normal gastric mucosa was anticipated. In BD there is an associated change in the morphology of the epithelium of the distal esophagus, where the stratified squamous epithelium is replaced with a metaplastic columnar epithelium of mixed morphologies (Peters, et al., 2014). Sections taken from tumor patients with BD showed a gain in AGS2 in the morphology of the cell type between sections (Figure 6, C and D). Examination of AGS2 expression in these sections showed expression of AGS2 in areas where the morphology had changed to simple columnar epithelium and to areas of gland like tissue. Expression appeared particularly high in the areas surrounding gastric mucosa cells suggesting that these cells may be expressing higher levels of AGS2 than those present in the normal gastric epithelium. Although not noted it would be particularly interesting to examine if these gastric mucosa cells were also expressing any mucins. If this was found to be true it would agree in part with the morphological changes present in BD may indicate a protective adaptation to increased acid secretion from GORD (Chowdhury, et al., 2017). Further a recent paper by Jiang et al suggest that AGS2 positive secretory cells may be involved in the transition to BD in the gastro-esophageal junction (Jiang, et al., 2017). Examination of AGS2 expression in OAC taken from patients with esophageal junction tumours showed high expression of AGS2 in areas of secretory like tissue in the same way as those in the BD tissue sections. There appeared to be no expression of AGS2 in the areas of esophageal tumour but these cells expressing

AGS2 cells represent adjacent to the tumor cells. Experiments with P21C found that direct upregulation of AGS2 in growing cells was possible by using pro-neoplastic factors (Dunbar, et al., 2016). In a similar way it could be possible that reversion of AGS2 may be a factor in the transformation of BO to OAC.

It would be useful to test further samples taken from patients, particularly a series of experiments on tissue samples taken from the same patients pre-BO and with BO to see if the apparent difference in expression of AGS2 between pre-BO and BO holds. In addition, it would be useful to examine those patients who have stable BO and those where BO develops into OAC to see if the tumors are upregulating AGS2 over time. It would be also useful to try to determine if there is a close relationship between AGS2 and other products either by co-localization or via hybridization for AGS2 and the various MUC genes using different fluorescent tags or colors.

It would also be useful to examine whether stress affects AGS2 expression in OE19 cells in culture. Evidence from Cohen and suggests that AGS2 downregulation occurs through a sulphuric acid intermediate and that high concentrations of H₂O₂ inhibit other formation (Cohen, et al., 2015). It would therefore be useful to test different concentrations of H₂O₂ to examine AGS2 expression and other formation in cells of the OE19 cells. In addition it would also be beneficial to expand the approach to include other reducing and stress-inducing agents, such as butylcyclohexyl which induces ER stress.

7.6 Conclusions

The overall aim of this thesis, was to examine the effects of mass labeling agents on integrase interaction using pseudotyped cell lines as a cellular model of HIV. Notably, only on GP120 and FcR1. This thesis found that GP120 was not expressed or not expressed at sufficient levels to be detected in the supernatant and that levels of gp120 were not expressed in CD4⁺ cells and coculture identified with the FcR1 (2A121014) antibody (Abcam). However, further experiments are required to validate GP120 expression levels with other. Considering AG22, this thesis has shown that AG22 is not expressed in the normal macrophage but is expressed in HD. Furthermore AG22 is associated closely with cancer cells in OAC (Figure 6, E and F). In OAC 18th AG22 was shown to form inducible dependent down as well as interactions with other, as yet unknown proteins. This suggests that AG22 plays a role in the pathogenesis of OAC and may be an important factor in the regulation of HIV in OAC, with the mechanism still to be determined.

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