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Bile Acids and Barrett's Oesophagus: The Development of a Functional siRNA Screen to Identify Novel Therapeutic Targets

Fiona M Behan BSc

A Thesis Presented to the University of Dublin for the degree of Doctor of Philosophy



PhD in Molecular Medicine:
From Molecules to Medicine

Supervisor: Prof. Dermot Kelleher

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Fiona M Behan

Summary

The development of oesophageal adenocarcinoma (OAC) occurs in a setting of chronic reflux known as gastro-oesophageal reflux disease (GORD) and with a recognised premalignant lesion, Barrett's oesophagus (BO). BO is characterised by the replacement of squamous epithelium of the distal oesophagus with a columnar epithelium. The presence of GORD is associated with an increased risk of developing BO which in itself carries a high risk for OAC development. Refluxed bile acids have been implicated in this process which is often referred to as the metaplastic-dysplastic-adenocarcinoma (M-D-A) sequence. OAC is an extremely aggressive cancer with a poor prognosis. Therapeutic options for this disease are limited, especially if diagnosed at the later stages. The main aim of this study was the identification of novel therapeutic strategies for the treatment of OAC through the development and implementation of a high throughput siRNA screening approach.

A gene expression microarray (GEM) analysis strategy was established combining clinical data from previously published studies with *in vitro* data generated in a novel study of the action of the deoxycholic acid (DCA) on oesophageal cells. This was performed to establish the molecular contribution of DCA to the M-D-A sequence. Approximately 33% of genes altered in OAC were regulated by DCA. Systems biology analysis identified cell proliferation and death as important processes regulated by these genes. The cell viability of oesophageal cell lines in response to DCA was established and it was determined that the dysplastic and adenomatous cell lines were resistant to DCA induced cell death.

A high throughput siRNA screening protocol was developed to facilitate the identification of modulators of this resistance to DCA induced cell death. A semi-automated siRNA transfection protocol using liquid handling robots was designed. B-cell lymphoma 2 like 2

(BCL2L2 aka BCL-XL) was developed as a positive control for this screen. An siRNA screen targeting G-protein coupled receptors (GPCRs) was completed. This siRNA screen identified interleukin 8 receptor alpha (IL8Rα), interleukin 8 (IL8) and frizzled homolog 1, 3 & 10 (FZD 1, FZD3, and FZD10) as potential modulators of resistance to DCA induced cell death. The simultaneous inhibition of BCL-XL, myeloid cell leukaemia sequence 1 (MCL1) and GATA binding protein 6 (GATA6) was established as a positive control in a screen examining the proliferative potential of GOhTRT cells. It was discovered during this preliminary work that combined inhibition of GATA6, BCL-XL and MCL1 substantially reduced cell viability.

Caudal type homeobox 2 (CDX2) is involved in the maintenance of intestinal epithelial homeostasis. Oesophageal tissue is normally negative for CDX2 expression but in the case of BO, CDX2 expression increases dramatically. As BO progresses into OAC, CDX2 expression decreases. This study has demonstrated that DCA suppresses CDX2 expression in OE33 oesophageal cancer cells and that this process is regulated by NF-κB. In a colon cancer setting, a possible role for CDX2 as a tumour suppressor gene has been hypothesised. This study suggests the possibility of a similar role for CDX2 in oesophageal adenocarcinogenesis. NF-κB is an ideal candidate to act as a positive control in an siRNA screen investigating the regulation of CDX2 expression.

This study has identified potential novel therapeutic targets and strategies for the treatment oesophageal cancer. This study has also established an innovative microarray analysis strategy capable of expanding on the potential impact of future microarray studies. A robust high throughput siRNA screening system has also been developed to facilitate large siRNA library screens and attempt to identify further novel therapeutic strategies for the treatment of oesophageal disease.

Dedication

To each and every individual that has shaped my life;

you made me who I am today,

I dedicate this you.

Acknowledgements

My eternal gratitude goes to -

My family - Dad, Mam, Trish, Peter and Maria (and Anna): you picked me up when I fell; you opened every door for me; you listened to my rants and gave me advice when I needed it, even if I didn't want it. For the food parcels, the roof over my head, the beer when I couldn't afford it; for always being at the end of the phone when I needed you, I am truly grateful. My only wish is that someday I can repay you for all you have done, but for now, you have my eternal thanks and the knowledge that I have finally finished college (almost!).

Ken - you have seen me at my worst, you have seen me at my best and everything in between yet you still hang around. You have put up with a lot over the past couple of years but you never failed to put a smile on my face. I look forward to spending many more happy moments with you for years to come.

My dear friends - to those who I have lost touch with and those who tried to lose touch with me: we've had some good times, we've had some bad times, here's to a future of more good than bad.

All my work buddies - Spaniard: for the all the entertainment in the lab and outside. Eugene: for all your help, advice, encouragement and Ken. The inhabitants of the boy's office, the girl's office and the other office: for answering all my stupid questions and listening to me talk about rugby 95% of the time.

The DK research group (past & present): Specifically to Dermot and Shane for all your supervision and guidance. Also to Aideen, Henry and Yuri: for sharing your knowledge and wisdom. Mary Kavanagh for all you tireless work and help whenever I needed it.

All the research groups in the IMM that have assisted me over the last three years - especially the Cell Signalling Group, HCA Suite, Dr. Stephen Smith and the Haemostasis & Thrombosis Groups

The Health Research board for funding this adventure; to Ruth, Darragh, Jenny, Emma, Muriel and Paul for sharing this adventure; to Ross, Sheila and Mike for keeping the adventure on the road.

The security staff, administration staff & Kylemore staff.

The Munster, Irish, Leinster, Ulster, Connacht, Newbridge & Lab Rats rugby teams: for the welcome distraction when I needed it, and holding the tackle bag when I needed an outlet (sorry ladies!).

My Godmother, Aunts, Uncles, relations & neighbours.

Those who I have forgotten today: Please accept my sincere apologies, writing this thesis appears to have affected my memory and my manners, so please don't hold it against me, hold it against the thesis.

Abbreviations

AGS	Human Gastric Adenocarcinoma Cell Line
AKT	v-akt Murine Thymoma Viral Oncogene Homolog 1
APC	Adenomatous Polyposis Coli
APS	Ammonium Persulfate
BAC	Bacterial Artificial Chromosome
BAX	BCL2-associated X protein
BCL2	B-cell CLL/lymphoma 2
BCL6	B-cell CLL/lymphoma 6
BCL-W	aka BCL2L2 - BCL2 like 2
BCL-XL	aka BCL2L1 - BCL2 like 1
BEBM	Bronchial Epithelial Cell Basal Medium
BisI	Bisindolylmaleimide I
BO	Barrett's Oesophagus
CA	Cholic Acid
Caco2	Colorectal Adenocarcinoma Cell Line
CCL25	Chemokine (C-C motif) ligand 25
CCND1	Cyclin D1
CDKN2A	Cyclin-dependent Kinase Inhibitor 2A aka p16
CDX1	Caudal Type Homeobox 1
CDX2	Caudal Type Homeobox 2
CER I	Cytoplasmic Extraction Reagent I
CER II	Cytoplasmic Extraction Reagent II
CIM	Intestinal Metaplasia of the Cardiac Mucosa
Ct	Cycle Threshold
DBS	Downstream Binding Site
DCA	Deoxycholic Acid
DHCA	Dehydrocholic Acid
DMSO	Dimethyl Sulfoxide
DRD1	Dopamine Receptor D1
EDTA	Ethylenediaminetetraacetic Acid
ERBB2	v-erb-b2 erythroblastic leukaemia viral oncogene homolog 2, neuro/ glioblastoma derived oncogene homolog (avian)
ERK	aka MAPK1 - mitogen-activated protein kinase 1
FCS	Fetal Calf Serum
FZD1	Frizzled Homolog 1 (Drosophila)
FZD10	Frizzled Homolog 10 (Drosophila)
FZD2	Frizzled Homolog 2 (Drosophila)
FZD3	Frizzled Homolog 3 (Drosophila)
GAPDH	Glyceraldehyde-3-phosphate Dehydrogenase
GATA6	GATA Binding Protein 6
GCDCA	Glycochenodeoxycholic acid
GDF15	Growth Differentiation Factor 15
GEM	Gene Expression Microarray

GEO	Gene Expression Omnibus
GLP1R	Glucagon-like Peptide 1 Receptor
GOhTRT	Dysplastic Oesophageal Cell Line
GORD	Gastro-oesophageal Reflux Disease
GPCRs	G-protein Coupled Receptors
GPR23	G-protein Coupled Receptor 23
GSK3α	Glycogen Synthase Kinase 3 alpha
HBSS	Hank's Balanced Salt Solution
HCA	High Content Analysis
HCT116	Colorectal Carcinoma
Het-1A	Normal Oesophageal Cell Line
HGD	High Grade Dysplasia
HIF1α	Hypoxia Inducible Factor 1, alpha subunit
HNF1α	HNF1 homeobox α
HNF4α	Hepatocyte Nuclear Factor 4, alpha
HT29	Colorectal Adenocarcinoma Cell Line
HT/HA	High Throughput/High Content
IL4	Interleukin 4
IL8	Interleukin 8
IL8Rα	Interleukin 8 Receptor α
IPA	Ingenuity Pathway Analysis
IM	Intestinal Metaplasia
LES	Lower Oesophageal Sphincter
MAP	Mitogen-activated Protein
MCL1	Myeloid Cell Leukaemia Sequence 1 (BCL2-related)
M-D-A	Metaplastic-Dysplastic-Adenocarcinoma
MDM2	transformed mouse 3T3 cell double minute 2
MEK1	aka MAP2K1 - Mitogen-activated Protein Kinase Kinase 1
mRNA	Messenger RNA
miRNA	microRNA
MTT	(3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide - Yellow Tetrazolium Salt
NER	Nuclear Extraction Reagent
NF-κB	Nuclear Factor of Kappa Light Polypeptide Gene Enhancer in B-cells
OAC	Oesophageal Adenocarcinoma
OCT1	Octamer-binding Transcription Factor 1
OE33	Oesophageal Adenocarcinoma cell line
OTP	ON-TARGET Plus
PARP	Poly (ADP-ribose)-polymerase-1
PCR	Polymerase Chain Reaction
PDT	Photodynamic Therapy
PI3K	Phosphoinositide-3-kinase, catalytic
PIK3CB	Phosphoinositide-3-kinase, catalytic, beta polypeptide
PIK3CG	Phosphoinositide-3-kinase, catalytic, gamma polypeptide
PKC	Protein Kinase C

PSG	Penicillin-Streptomycin-L-Glutamine
PTEN	Phosphatase and Tensin Homolog
PTGER4	Prostaglandin E Receptor 4 (subtype EP4)
PTGS2	Prostaglandin-endoperoxide Synthase 2 (prostaglandin G/H synthase and cyclooxygenase)
QhTRT	Oesophageal Metaplastic Cell Line
RAR	Retinoic Acid Receptor
RB1	Retinoblastoma 1
RELA	v-rel reticuloendotheliosis viral oncogene homolog A (avian) aka p65 subunit of NF-κB
RISC	RNA-induced silencing complex
RFU	Relative Fluorescent Units
RGS16	Regulator of G-protein Signalling 16
RNAi	RNA interference
RORα	RAR-related orphan receptor alpha
ROS	Reactive Oxygen Species
SERPINA1	Serpin Peptidase Inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 1
SFRP	Secreted Frizzled-related Protein
shRNA	short hairpin RNA
siNT or NT	Non-targeting
siRNA	small interfering RNA
SKGT4	Oesophageal Adenocarcinoma Cell Line
TAS2R39	Taste Receptor, type 2, member 39
TCF4	Transcription Factor 4
TEMED	Tetramethylethylenediamine
TP53	Tumour Protein p53
TR/RXR	Thyroid Hormone Receptor/Retinoid X Receptor
TRIB3	Tribbles Homolog 3 (Drosophila)
UTR	Untranslated Region
VEGF	Vascular Endothelial Factor
VIL1	Villin 1
WIF1	Wnt Inhibitory Factor 1
Wnt1	Wingless-type MMTV Integration Site Family, Member 1
WR	Working Reagent

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Chapter 1

General Introduction

1 General Introduction

1.1 Overview

Variations in the historical levels of exposure to disease associated risk factors have resulted in different trends across time and geographic locations for the occurrence of different cancers. Currently, the incidence rates of oesophageal adenocarcinoma (OAC) have steadily increased over the last number of decades in Western countries whereas other oesophageal carcinoma rates have remained stable or decreased (Parkin, 2005, Sundelof et al., 2002). This may be a result of increased exposure to the primary risk factor linked with OAC which is the development of chronic reflux known as gastro-oesophageal reflux disease (GORD) and associated Barrett's oesophagus (BO), a premalignant condition. BO involves the replacement of the normal squamous epithelium with a columnar epithelium containing goblet cells. Oesophageal adenocarcinoma is an extremely aggressive cancer with a poor prognosis. Although increased survival rates have been observed over recent decades, the 5 year survival rate is predicted to be less than 15% (Sundelof et al., 2002). Surveillance programs have been introduced in an attempt to detect the disease at early, more treatable stages. Currently, surgical resection is one of the best curative treatments available, however not all patients are suitable for this option. Earlier diagnosis will allow for the implementation of other interventions such as endoscopic mucosal resection, photodynamic therapy (PDT), radiofrequency ablation, chemotherapy radiotherapy or chemoradiotherapy (Yousef et al., 2008). Over 50% of patients demonstrate no response to combined neoadjuvant chemotherapy and radiation therapy with only 25% displaying a complete pathologic response (Abdel-Latif et al., 2004).

1.2 Barrett's oesophagus

Barrett's oesophagus (BO) occurs when the normal squamous epithelium of the oesophagus is replaced by a metaplastic columnar epithelium containing goblet cells.

Damaged oesophageal mucosa heals with the replacement of normal epithelium with a more resistant columnar epithelium (Jankowski et al., 1999). Barrett's oesophagus is named after Norman Barrett, an oesophageal surgeon who was prominent in London in the mid-20th century. The main events in the history of BO are summarised in Table 1.1. It is estimated that there are 2.5 million Americans currently living with BO (DeMeester et al., 2001). There have been a number of changes to the definition of BO over the last six decades, the most important of these is the post 1994 definition that includes columnar epithelium <3cm in the definition of BO (Table 1.1). It is still believed there has been an increase in the absolute number of cases of BO diagnosed each year as opposed to the increase in incidence being a consequence of more rigorous screening, changes in the definition of the disease and improved methods of detection methods (Prach et al., 1997).

BO is associated with an increased risk of developing OAC compared to normal age matched controls. The elucidation of the molecular mechanisms underlying the development of BO is extremely important (Falk, 2002). A greater understanding of these mechanisms may lead to the discovery of biomarkers to aid in the diagnostic process. Improved methods of diagnosis allowing for earlier detection of disease should lead to improved prognosis as there is an exact correlation between the stage of the oesophageal carcinogenic sequence at which diagnosis is made and survival rates. (Ong et al., 2010). It has been demonstrated that the greater the length of columnar mucosa that is present in the oesophagus, the greater the chance of discovering intestinal-type metaplasia (IM) of the oesophagus. Also, if IM is to be found in the oesophagus, the most likely location of the IM is at the most proximal end of the cardiac mucosa adjacent to the squamocolumnar junction. This area of the mucosa is the furthest columnar epithelium from the stomach and would have the highest pH level. This increase in pH facilitates an increase in cellular

differentiation and a decrease in proliferation, allowing for the development of intestinal-like mucosa (Zhou et al., 2009).

Table 1.1: Summary of the history of BO described in Spechler *et al* (2010).

1839	Thought to be first report of peptic ulcer in the oesophagus by German pathologist, Albers.
1906	Description of 3 cases of 'peptic ulcer of the oesophagus' in Boston by Wilder Tileston
1950	Barrett describes ulcerated portion of the oesophagus, argues it is tubular element of the stomach tethered to a congenitally short oesophagus.
1951	Bosher & Taylor describe intestinal-type goblet cells in columnar-lined oesophagus.
1952	Morson & Belcher describe an adenocarcinoma with intestinal type mucosa containing goblet cells.
1953	Allison & Johnstone describe 7 patients with 'oesophagus lined with gastric membrane'; refuted presence of gastric tissue; proposed condition be called 'Barrett's ulcers'.
1970s	Well established that BO is associated with GORD and hiatal hernia, both can obscure endoscopic landmarks of the gastroesophageal junction resulting in biopsies being taken from below the gastroesophageal junction giving false diagnosis of BO; development of pH monitoring techniques using pH monitors positioned in the oesophagus.
1976	Paull *et al* described three types of oesophageal columnar epithelia: a junctional epithelium; a gastric fundic-type epithelium and intestinal-type metaplasia.
1980s	It was decided that arbitrary lengths of columnar lining were required for a diagnosis of BO as Hayward described up to 2cm of cardia-type epithelium in 'normal' individuals. Also required were symptoms and endoscopic signs of GORD.
Late 1980s	The most common type of BO emerges as intestinal-like epithelium, which also appeared to be associated with cancer development.
Early 1990s	The presence of intestinal-type metaplasia was required for BO diagnosis. Biopsies would not be taken unless patient suffered from GORD.
1994	Spechler argued that columnar epithelium less than 3cm was significant; also demonstrated that GORD was not a reliable marker for intestinal metaplasia.
Late 1990s	BO is characterised as long segment (metaplastic epithelium >3cm) or short segment (<3cm). Overall definition of BO was the presence of intestinal epithelium of any length in patients with or without GORD.
Currently	The AGA Institute recommends that BO should be defined as 'the condition in which any extent of metaplastic columnar epithelium that predisposes to cancer development replaces the stratified squamous epithelium that normally lines the distal oesophagus'.

BO, IM and cardiac mucosa all appear to be linked by reflux. Bile appears to drive intestinalisation and acid seems to promote the development of cardiac mucosa. The length of IM is inversely correlated with lower oesophageal sphincter (LES) function and correlates significantly with the amount of time that the tissue spends exposed to a pH of less than 4 (DeMeester and DeMeester, 2000).

BO is thought to develop in two distinct steps: transdifferentiation of squamous mucosa to cardiac mucosa followed by intestinalisation of the cardiac mucosa. The length of cardiac mucosa increases with exposure to reflux. The presence of cardiac mucosa indicates an alteration in differentiation of oesophageal squamous cells towards a more acid resistant columnar mucosa. The second step in the development of oesophageal OAC is the intestinalisation of the cardiac or columnar mucosa. Bile salts have been demonstrated to induce intestinal transcription factors in oesophageal cells and as such are candidate modulators of intestinalisation (DeMeester et al., 2001). It has also been established that the total bile salt concentration in patient groups with erosive esophagitis and BO were significantly higher than in a group of patients with minimal injury (Nehra et al., 1999).

The exact mechanism involved in the replacement of oesophageal squamous cells with a columnar type epithelium during the development of BO is not currently well understood. A number of theories have been proposed as to the origins of BO. These theories include the creeping of cardia mucosa, the re-differentiation of the normal squamous epithelium and the clonal expansion of cells originating from the oesophageal gland ducts. The creeping of cardia mucosa is probably the most unlikely of these theories as normal squamous islands have been identified between the cardia and the BO mucosa. An uninterrupted progression from cardia to BO mucosa would be expected if creeping of the cardia mucosa was responsible for the development of BO (Maley, 2007). Leedham *et al*

(2008) microdissected and genetically analysed individual oesophageal gland ducts to establish a high resolution insight as to the source of both Barrett's metaplasia and neo-squamous epithelium. This study demonstrated that observed heterogeneity in BO is a result of independent clones developing from a progenitor cell located in the squamous gland ducts. The presence of normal squamous areas in the mosaic pattern of BO probably arises from wild-type ducts (Leedham et al., 2008). The potential role of stem cells as the progenitor cells arising from individual crypts is described in more detail in section 1.3.1. It has also been postulated that wound healing in response to mucosal injury caused by bile salts may contribute to the development of BO. The process of wound healing includes a number of phenotypic changes in a cell that are similar to the those acquired during the development of cancer known as the hallmarks of cancer (Hanahan and Weinberg, 2000). Importantly, cells involved in the wound healing process do not have limitless replicative potential. The development of a survival or proliferative competitive advantage in these cells may contribute to the development of malignancy. Concurrent inflammatory signals as a result of bile acid exposure may result in an increase in proliferation and mutagenesis. Repeated cycles of bile acid induced damage would naturally select for cells which have developed survival and reproductive advantages, which may allow for the transformation of normal squamous mucosa into columnar mucosa or intestinal metaplasia (Maley, 2007, Dvorakova et al., 2005).

Possible intermediate cells in the development of BO have also been identified at the squamocolumnar junction. These cells express microvilli, intercellular ridges and surface microridges. This combination of features is unique and may point to the identification of an intermediate stage between development of columnar mucosa and intestinalisation of that columnar mucosa (DeMeester et al., 2001). However, these cells appear to have been described in only one publication and require further investigation.

Inflammation appears to be a key element in progression through the oesophageal carcinogenic sequence and as such may be a useful target as a therapeutic option for BO. COX2 inhibitors held some promise in halting the progression of oesophageal malignancies however side effects involving cardio toxicity were observed which removed the potential for this therapeutic strategy (Bresalier et al., 2005). The largest prospective intervention study ever carried out examining chemoprevention in patients with BO is currently being carried out in the UK. This study is known as the ASPECT study. Aspirin and esomeprazole, a proton pump inhibitor, are being used to target mortality and decrease the number of patients that progress from BO to OAC or high grade dysplasia. Biopsies will be obtained every 2 years from patients enrolled in this study, making it possible to characterise the expression of p16, p53 and ploidy changes. The protein expression levels of CDX2, COX2, PKCε and MCM2 will also be assessed followed each biopsy (Zagorowicz and Jankowski, 2007). Expression levels of the most important players in oesophageal adenocarcinogenesis can be assessed in each individual case following each routine biopsy. This will provide a valuable insight into the molecular mechanisms behind oesophageal adenocarcinogenesis.

1.2.1 Caudal type homeobox transcription factors and Barrett's oesophagus

The CDX family of proteins contain a homeobox region and a consensus sequence of 60-63 amino acids which acts as a DNA binding motif. This homeobox allows the members of the CDX family (CDX1, CDX2 and CDX4) to act as transcriptional regulators for downstream target genes (Beck, 2004). Target genes of CDX2 include mucin, sucrose isomaltase, villin 1, defensin 5 and alkaline phosphatase (Huo et al., 2010). These genes are all used as intestinal markers as CDX2 is normally located exclusively in the adult human small and large intestine (Huo et al., 2010). This family of proteins has an important role in embryonic development as they facilitate the differentiation of the

endoderm into intestine tissue types and establish a functional axis along the entire gut (Souza et al., 2008). CDX2 appears to have an essential role to play in development, as the absence of Cdx2 in $Cdx2^{-/-}$ mice results in embryonic lethality (Bonhomme et al., 2003). $Cdx2^{+/-}$ mice are viable however they are susceptible to the spontaneous occurrence of multiple colonic polyps in the proximal colon (Kim et al., 2004). CDX2 may be relevant in BO as this molecule has been associated with transition from GORD to BO (Eda et al., 2003).

CDX2 functions as a master regulatory gene with responsibility for axial patterning of the alimentary tract during embryonic development. CDX2 is also involved in the maintenance of adult intestinal epithelial homeostasis through the transcriptional regulation of multiple genes involved in cell differentiation, cell proliferation, adhesion and apoptosis (Vaninetti et al., 2009). Differentiation and apoptosis are stimulated and cell proliferation is inhibited in intestinal cell lines with forced expression of CDX2 (Kim et al., 2002). Oesophageal tissue is normally negative for CDX2 expression but has been detected in BO cells indicating a potential role for CDX2 in the development of BO from normal squamous epithelium (Groisman et al., 2004).

1.2.1.1 CDX2 expression

The expression level of CDX2 varies greatly throughout the oesophageal carcinogenic sequence. Normal squamous oesophageal cells are generally found to be negative for CDX2 expression (Groisman et al., 2004). However, normal squamous cells of BO patients have reacted positively for CDX2 expression without exhibiting any phenotypic morphological changes which would be expected from CDX2 positive metaplastic cells. This would indicate that CDX2 induction may be an early event in development of columnar metaplasia in the oesophagus (Hu et al., 2007). CDX2 has also been identified in

non-goblet containing columnar cells adjacent to goblet cell containing intestinal metaplasia in oesophageal tissue, a further indication that CDX2 is an early event in oesophageal tissue transformation (Groisman et al., 2004). An increase in CDX2 expression is observed as cells progress through the carcinogenic sequence from normal squamous to cardiac mucosa to Barrett's oesophagus (Vallbohmer et al., 2006). Interestingly, as Barrett's oesophagus develops into adenocarcinoma through varying levels of dysplasia, CDX2 expression levels decrease as the grade of dysplasia increases and adenocarcinoma develops (Phillips et al., 2003, Vaninetti et al., 2009).

This decrease in CDX2 expression is also observed in colorectal tumours where a loss in CDX2 is observed in the later stages of colorectal carcinogenesis, specifically high grade and invasive carcinomas (Ee et al., 1995). It is postulated that CDX2 has a tumour suppressor function in colon carcinogenesis as a reduction in CDX2 expression facilitates tumour progression. There is currently no evidence describing a role for CDX2 in tumour initiation (Bonhomme et al., 2003). Further evidence that CDX2 may function as a tumour suppressor arises from its ability to inhibit cell proliferation of HT-29, IEC-6 and Caco2 cell lines (Kim et al., 2004, Guo et al., 2004). CDX2 inhibits COX2, a potent enhancer of carcinogenesis and is downregulated by the oncogene *ras* in Caco2 and HT29 cells (Kim et al., 2004) (Guo et al., 2004). CDX2 expression appears to be required for the development of Barrett's oesophagus but conversely, CDX2 expression may interfere with the development of carcinogenesis (Guo et al., 2004).

1.2.1.2 CDX2 and bile acids

The secondary bile acid deoxycholic acid (DCA) has been shown to downregulate CDX2 promoter activity in HT29 cells as does the bile acid chenodeoxycholic acid (CDCA). In contrast, dehydrocholic acid (DHCA) and cholic acid (CA) induce promoter activity in this

cell line (Kazumori et al., 2006). The induction of CDX2 by bile salts and acid has been demonstrated in cells derived from GORD patients with associated Barrett's oesophagus, whereas GORD patients without Barrett's oesophagus do not exhibit an induction in CDX2 expression in response to bile salts and acid (Huo et al., 2010). Bile salts have the capacity, through altering the expression level of CDX2, to affect a broad range of cellular process such as proliferation, differentiation, adhesion and apoptosis (Vaninetti et al., 2009). Elucidation of the signalling pathways involved in bile salt regulation of CDX2 transcription has huge potential in the quest to identify novel therapeutic targets.

1.2.1.3 Regulation of CDX2

A number of molecules have been associated with CDX2 transcriptional regulation such as caudal type homeobox 1 (CDX1), hepatocyte nuclear factor 4, alpha (HNF4α), NF-κB and CDX2 itself. NF-κB, a proinflammatory transcriptional regulator, has been implicated in the transcriptional regulation of CDX2 through identification of two NF-κB binding sites in the promoter region of CDX2. Mutation of each of these binding sites individually resulted in a partial ablation of CDX2 induction in response to DHCA in HT29 cells with a complete elimination of CDX2 induction following mutation of both NF-κB binding sites simultaneously (Kazumori et al., 2006). A binding site for the octamer-binding transcription factor 1 (Oct1) has been identified on the mouse Cdx2 promoter region (Jin and Li, 2001). Transient transfection of a CDX1 expression vector into Het-1A and OE33 cells resulted in an increase in expression of a CDX2 reporter vector, suggesting a regulatory role for CDX1 in CDX2 expression (Kazumori et al., 2009). The CDX2 promoter also possesses a downstream binding site (DBS) which facilitates autoregulation (Kazumori et al., 2009, Xu et al., 1999). Post-translational regulation of CDX2 has also been suggested, specifically by phosphorylation of a serine residue in the activation domain (Rings et al., 2001).

1.2.1.4 NF-κB as a regulator of CDX2

One of the primary regulators of CDX2 that has been discovered is NF-κB. NF-κB expression levels increase with progression through the oesophageal carcinogenic sequence with little NF-κB evident in normal squamous cells and maximal expression in adenocarcinoma (Abdel-Latif et al., 2009). Inhibition of NF-κB in cells derived from GORD patients with associated Barrett's oesophagus prevented an induction of CDX2 expression in response to bile salt and acid exposure (Huo et al., 2010). DCA exposure increases NF-κB in OE19 cells assessed by nuclear translocation of NF-κB and the binding of the p50 subunit to the CDX2 promoter (Debruyne et al., 2006). The p50 and the p65 subunits of NF-κB have opposing effects on CDX2 expression. The binding of the p50 homodimer to the CDX2 promoter region results in an induction of CDX2 expression, whereas p65 inhibits the action of p50 with the formation of a p65/p50 heterodimer which results in an inhibition of CDX2 expression (Huo et al., 2010). The p65 subunit is predominately located in the proliferative zone of intestinal epithelium with the p50 subunit located in the differentiating zone (Kim et al., 2002).

1.2.1.5 Other potential regulators of CDX2 expression

GATA binding factor 6 (GATA6), hepatocyte nuclear factor 4 (HNF4α), transcription factor 4 (TCF4) and β-catenin have been suggested as promoters of CDX2 expression in mice, both individually and synergistically (Benahmed et al., 2008). The mechanism by which this occurs appears to be complex and will require further work in both mice and human cells to confirm. HIF-1α has also been suggested as a negative regulator of CDX2, suppressing CDX2 expression (Zheng et al., 2010). Interestingly, HIF-1α expression decreases as normal squamous cells become intestinalised and increases as OAC develops (Griffiths et al., 2007). This finding indicates a possible role for HIF-1α in the regulation

of CDX2 in the oesophagus as CDX2 expression in the oesophagus inversely correlates with this negative regulator (Phillips et al., 2003).

1.2.2 Wnt signalling and Barrett's oesophagus

One of the normal functions of the Wnt signalling pathway is the morphogenesis of the gastrointestinal tract (Korinek et al., 1998). Aberrant activation of the Wnt signalling pathway has recently been identified during the development of BO (Clement et al., 2006). β-catenin, a downstream target of Wnt signalling has also been implicated in the development of BO, even though mutations in the β-catenin gene are rarely found in oesophageal malignancies (Wijnhoven et al., 2000). β-catenin is normally mutated on exon 3 which prevents the accumulation of β-catenin in the nucleus. This mutation has not been detected in OAC. Components of the Wnt signally pathway such as wingless-type MMTV integration site family, member 1 (Wnt1) or glycogen synthase kinase 3 alpha (GSK3α) may inhibit β-catenin nuclear accumulation. Alternatively, microRNAs have been implicated in this pathway. Specifically, miR21, miR200a and miR18 have been demonstrated to downregulate β-catenin whereas miR315 and miR135a/b upregulate β-catenin (Huang et al., 2010). Activated β-catenin accumulates in the nucleus where it acts as a transcription factor inducing the expression of tumour growth promoting genes (Cavallo et al., 1998). As the β-catenin gene is not itself mutated, it is postulated that altered expression of β-catenin during the development of OAC is a consequence of alterations to the upstream control mechanisms involved in the regulation of β-catenin activity (Figure 1.1). Wnt signalling is initiated by the binding of secreted Wnt proteins to transmembrane receptors on the cell surface (Frizzled receptors (FZD) and the single-pass transmembrane low-density lipoprotein receptor-related proteins 5 and (LRP5/6)) (Tamai et al., 2000). This signalling pathway results in the stabilisation of β-catenin and its accumulation in the nucleus and the activation of target genes (Cavallo et al., 1998).

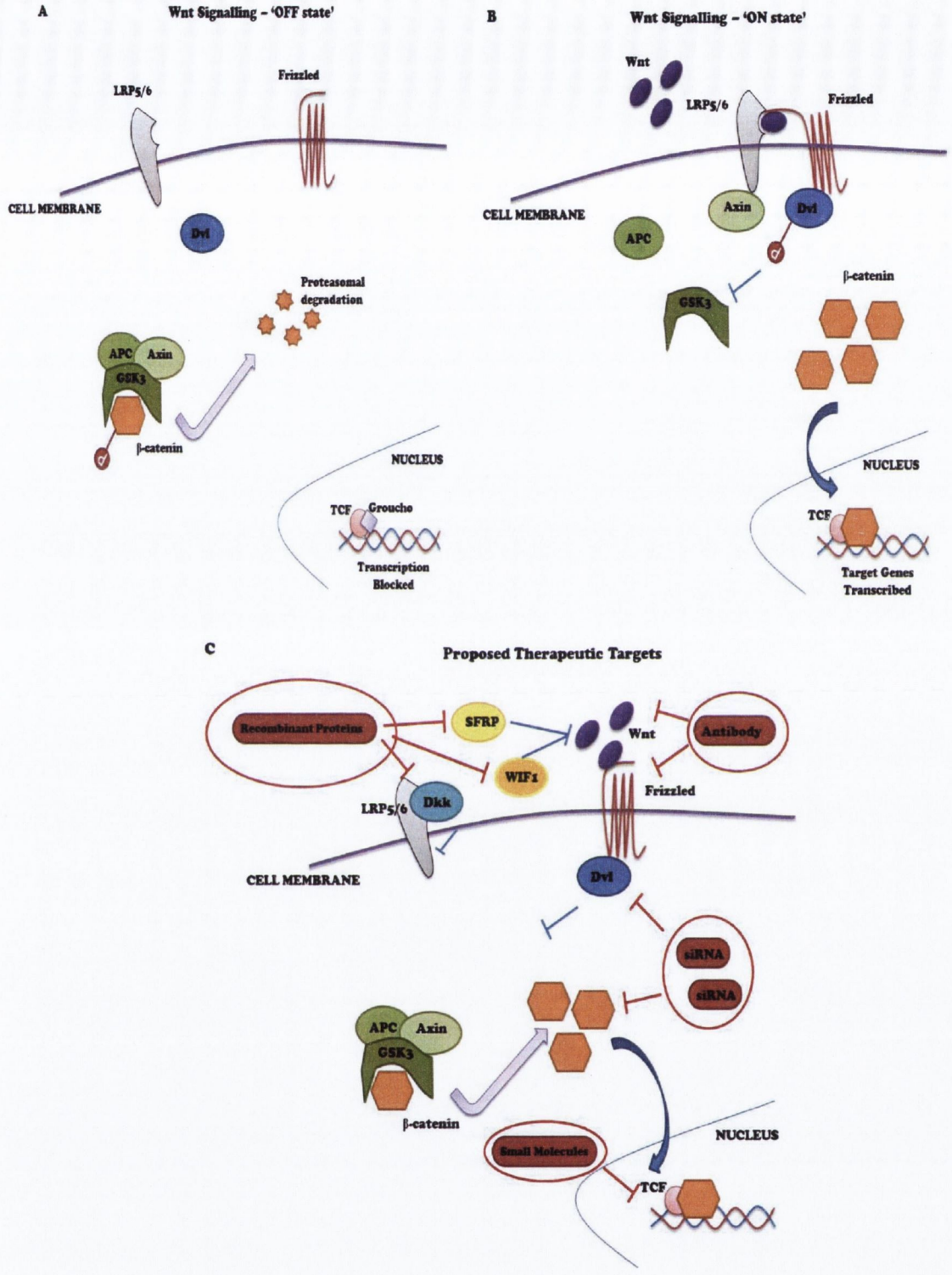


Figure 1.1: Wnt signalling pathway and potential therapeutic targets. (A) When the Wnt signalling pathway is in the 'OFF state', β-catenin is degraded which prevents its accumulation in the nucleus and target genes are not transcribed. (B) The binding of secreted Wnt proteins controls the activation of the Wnt signalling pathway. When the pathway is activated, β-catenin can accumulate in the nucleus, resulting in the transcription of target genes. (C) Proposed therapeutic strategies in relation to this pathway can target a number of elements as highlighted by the red circles. Small molecular inhibitors, siRNA molecules, recombinant proteins and antibodies are the proposed mechanisms to disrupt this pathway for the treatment of oesophageal disease *(modified from* (Clément et al., 2007)).

In the case of oesophageal malignancies, inhibitors of secreted Wnt proteins, secreted frizzled related protein (SFRP) and Wnt inhibitory factor-1 (WIF-1) are downregulated following epigenetic methylation. This results in an increase in the activity of the secreted Wnt proteins, an upregulation of the Wnt signalling pathway and an accumulation of β-catenin in the nucleus. Suggested therapeutic strategies to target this pathway include the use of recombinant proteins to rescue the expression of the SFRP and WIF-1 and the use of antibodies, siRNA molecules or small molecular inhibitors to inhibit the FZD and the LRP5/6 receptors (Clément et al., 2007) (Figure 1.1). Wnt signalling is also controlled by a number of microRNAs such as miR-8 and miR200a (Huang et al., 2010). An elegant study investigating epithelial stem cell development and airway regeneration has identified a novel link between the GATA6 transcription factor and Wnt signalling. GATA6 was demonstrated to regulate FZD2 gene expression. FZD2 then functions as a negative regulator of canonical Wnt signalling. Loss of GATA6 activity caused an increase in proliferation and an increase in differentiation. The importance of this regulation relates to the balance between progenitor expansion and epithelial differentiation (Zhang et al., 2008). Further investigation into the role of GATA6 and Wnt signalling in an oesophageal setting merits further investigation.

1.3 Oesophageal carcinogenic sequence

Development of GORD is believed to be the first event in the sequential occurrence of a number of defined precursor conditions leading to OAC known as the oesophageal carcinogenic sequence or the metaplastic-dysplastic-adenocarcinoma (M-D-A sequence) (Jankowski et al., 1999, Chen and Yang, 2001). Epidemiology studies have concluded that the presence of intestinal metaplasia and BO increases the chance of developing oesophageal adenocarcinoma by 30 to 125 times when compared to an age-matched population (Yousef et al., 2008).

The presence of a non-intestinal type columnar epithelium has been identified and is referred to as cardiac or junctional type mucosa and is thought to be a precursor to Barrett's oesophagus. Cardiac mucosa is important as this type of epithelium is believed to be the only mucosal type that progresses to intestinal metaplasia (DeMeester and DeMeester, 2000). Intestinal metaplasia of the cardia mucosa (CIM) appears to be more resistant to the harmful effects of reflux than squamous mucosa as inflammation is greatly reduced in CIM (DeMeester et al., 2001). Currently, determination of the stage of the M-D-A sequence present at the time of diagnosis is of extreme important as this is the best predictor of prognosis currently available (Chen and Yang, 2001).

Progression through the M-D-A sequence requires the accumulation of a number of genetic and epigenetic abnormalities (Figure 1.2). It is believed that an increased understanding of the molecular events that occur during oesophageal adenocarcinoma development will result in considerable advances in the treatment of this cancer. Elucidation of the underlying molecular mechanisms involved in the M-D-A sequence will present novel therapeutic targets to interfere with the early development of malignant cells and subsequent growth, invasion and metastatic potential (DeMeester et al., 2001). The estimated risk of progression to cancer associated with each stage of the oesophageal adenocarcinoma are 0.5% in patients with non-dysplastic Barrett's, 13% in patients with low grade dysplasia and 40% in patients with high grade dysplasia (Ong et al., 2010).

Development of metaplasia in the oesophagus involves the dedifferentiation of squamous epithelium into columnar epithelium (Milano et al., 2007) and occurs in the setting of GORD (Lagergren et al., 1999). Repeated episodes of reflux result in exposure of the oesophagus to damaging exogenous agents contained in refluxate. Components of the refluxate that are specifically implicated in causing mucosal injury include gastric acid and

bile salts which directly influence intestinal metaplasia development and progression to neoplasia in the oesophagus (Vaezi and Richter, 1996).

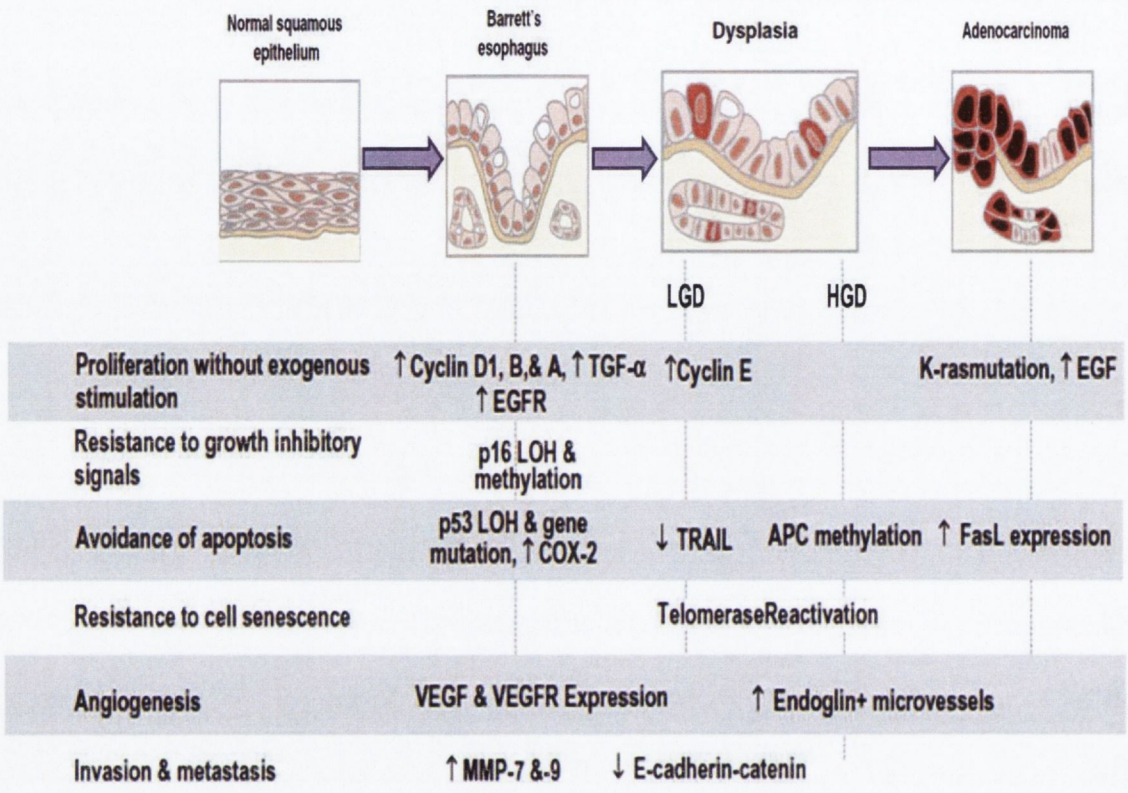


Figure 1.2: The major genetic events involved in progression through the oesophageal carcinogenic sequence. The oesophageal carcinogenic or M-D-A sequence is a well characterised stepwise process by which normal squamous epithelium transforms into oesophageal adenocarcinoma. A number of important genetic alterations are highlighted which occur during progression through this sequence which lead to the acquisition of the hallmarks of cancer (*modified from (Clément et al., 2007, Zhang et al., 2009b)).*

The levels of exposure to gastric acid and bile salts increase during progression through the oesophageal M-D-A sequence (DeMeester et al., 2001). The resulting injury to the oesophageal mucosa often heals with a metaplastic process where intestinal-type columnar cells replace the damaged normal squamous epithelium (Spechler, 2002). The severity of damage to the oesophageal mucosal layer is increased in patients with both gastric and duodenal components in the refluxate compared to patients with gastric juices alone (Kauer et al., 1997). The most important risk factor in the development of Barrett's oesophagus is the presence of reflux, not age (DeMeester et al., 2001). It has been demonstrated that

increased risk of development of oesophageal adenocarcinoma is also associated with more frequent, more severe and longer lasting the symptoms of reflux (Lagergren et al., 1999). There is a strong correlation between the concentration of bile acids in refluxate and the degree of mucosal injury. The concentration of bile acids in patients with erosive oesophagitis and BO were significantly greater than those in patients with minimal injury (Nehra et al., 1999). Bile reflux is also the main predisposing factor for Barrett's oesophagus (Vaezi and Richter, 1996). Barrett's oesophagus is implicated in the development of oesophageal adenocarcinoma as virtually all cases of oesophageal adenocarcinoma arise from Barrett's oesophagus (Wild and Hardie, 2003).

1.3.1 Stem cells and progression through the oesophageal carcinogenic sequence

The contribution of stem cells to the development and progression of various cancers has received increased attention. Stem cells isolated from a colon carcinoma have been found to be both necessary and sufficient for the initiation of tumour growth in immunodeficient mice. These tumour stem cells also appear to protect themselves from apoptosis through the production of Interleukin 4 (IL4) (Todaro et al., 2007). This resistance to apoptosis may result in resistance to cell death induced by radiation or chemotherapy, contributing to a poorer response to therapy and a poorer prognosis. In the case of oesophageal carcinogenesis, animal models involving dogs and rats concluded that columnar epithelium arises from a pluripotent stem cell present in the oesophageal glands (Miyashita et al., 2006). Further evidence of stem cell involved in OAC development was demonstrated in a study of the expression of the intestinal stem cell marker Lgr5 in BO. Increasing levels of Lgr5 positive stem cells were associated with BO, HGD and OAC with the highest intensity in OAC associated with the poorest survival rates (Becker et al., 2010). Musashi-1, also an intestinal stem cell marker, increasing during the development of OAC (Bobryshev et al., 2010). Further evidence of a role for Musashi-1 positive cells in tumour

promotion was obtained using siRNA targeting of this gene. The specific inhibition of this gene resulted in tumour growth arrest, reduced cell proliferation and increased apoptosis in HCT116 colon adenocarcinoma xenografts in nude mice (Sureban et al., 2008). Theoretical support for a role for stem cells in development of cancers of the gastrointestinal tract lies with the fact that intestinal stem cells have a sufficiently longer life to accumulate the necessary genetic abnormalities for cancer development when compared to other intestinal cell types. It has also been suggested that pathways essential for the survival of cancer stem cells have also been implicated in intestinal carcinogenesis. These pathways included the Wnt/APC/β-catenin, PI3K/PTEN and BMP signalling pathways (Bobryshev et al., 2010). Further evidence is required to gain a greater insight into the role of cancer stem cells in the tumourigenesis and is of extreme importance as cancer therapies need to be developed to target these cells to prevent tumour regrowth and metastasis. Solid tumours are predicted to have only a small portion of cells that could be referred to as cancer stem cells. These are cells that have the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise solid tumours (Clarke et al., 2006).

Bone marrow associated stem cells have also been associated with carcinogenesis, specifically in the development of oesophageal adenocarcinoma. Irradiated female rats injected with bone marrow from male rats developed intestinal metaplasia (IM) following an oesophagojejunostomy. Cells from this IM were shown to contain a Y chromosome, suggesting that circulating bone marrow could contribute to the development of oesophageal cancer (Sarosi et al., 2008). These stem cells utilise the local tissue environment to establish designated cell lineages specific to their location. Atypical environmental conditions found in oesophageal tissue exposed to chronic reflux may contribute to the differentiation of bone marrow derived cells into non-squamous tissue.

The plasticity of these bone marrow derived cells has been demonstrated in patients who have undergone gender mismatched bone marrow transplants (Li et al., 2006).

1.3.2 Molecular and genetic events involved in progression through the M-D-A Sequence

The main genetic changes that occur during development of oesophageal adenocarcinoma include loss of p16, loss of p53, increase in cyclin D1, induction of aneuploidy and the losses of the retinoblastoma (Rb), deleted in colonic cancer (DCC) and adenomatous polyposis coli (APC) loci (Jenkins et al., 2002). These molecules are associated with the control of the cell cycle and as such, aberrant expression can be highly deleterious for the cell.

The p53 protein controls progression through the cell cycle and promotes apoptosis if DNA damage is detected and as such is an extremely important molecule. The loss of p53 has been detected at all stages of the M-D-A sequence, suggesting a competitive advantage for cells with decreased functionality of this protein (Jenkins et al., 2002). Some clones exhibiting loss of p53 have been demonstrated to be resistant to PDT (Krishnadath et al., 2000). The loss of chromosome 17p, the location of the p53 gene, is the most common lesion in OAC, indicating the importance of loss of this molecule in progression through the M-D-A sequence (Reid et al., 2001).

Loss of expression of p16 is important in the progression through the M-D-A sequence as this gene is a tumour suppressor gene. The p16 protein is involved in the regulation of progression through the cell cycle by the suppression of retinoblastoma phosphorylation which results in the blocking of the cycle. The reintroduction of p16 into malignant cells with loss of expression resulted in a resensitisation of these cells to anchorage dependent

apoptosis thus reducing the metastatic potential of these cells (Rocco and Sidransky, 2001). The hypermethylation of p16 has been detected in both BO and ADC. Multivariate analysis has revealed that an increase in the methylation of p16 is associated with an increased risk in progression through the M-D-A sequence (Schulmann et al., 2005). The loss of p16 expression occurs so early in the M-D-A sequence that it is unsuitable as a biomarker for progression (Ong et al., 2010).

An increase in oxidative damage has also been associated with progression towards adenocarcinoma development (Goldman et al., 2010). Aneuploidy has also been correlated with progression from Barrett's oesophagus to oesophageal adenocarcinoma (Reid et al., 2001). The transcription factor NF-κB has also been implicated in progression through the M-D-A sequence as hyperploidy of chromosome 4, the location of the NF-κB, has been identified in nearly all metaplastic, dysplastic and neoplastic specimens examined (Jenkins et al., 2004). NF-κB expression has been detected to increase during oesophageal adenocarcinogenesis and is thought to be involved in conferring resistance to chemotherapy. OAC without expression of NF-κB is more susceptible to neoadjuvant therapy as such may act as a predictor of response to treatment (Abdel-Latif et al., 2004). NF-κB is thought to contribute to resistance to cancer treatment through the induction of expression of anti-apoptotic genes such as cellular inhibitors of apoptosis 1 and 2 (c-IAP1, c-IAP2), TNF receptor associated factors (TRAF1, TRAF2) and p53 (Yamamoto and Gaynor, 2001)

1.3.3 Clonal expansion

Progression through the oesophageal M-D-A sequence resulting in the development of oesophageal adenocarcinoma involves the selection of transformed clones that have acquired significant advantages over normal cells (Maley, 2007). The most competitive

clones will have adapted most successfully to the abnormal environment caused by chronic reflux. Clonal diversity develops in a complex, non-linear fashion and requires a number of significant molecular modifications (Barrett et al., 1999) (Figure 1.3). This simultaneous development of a number of diverse abnormal clones creates a mosaic pattern of metaplastic fields which will have varying degrees of malignant potential determined by the specific genetic aberrations that have been acquired (DeMeester et al., 2001). Mutations rates will also increase during sequence progression as the level of genetic instability increases (Breivik, 2005).

The expansion of mutated malignant clones, with selective advantage over competing squamous mucosa, has been proposed as a mechanism by which normal oesophageal mucosa is replaced by metaplastic and adenomatous tissue. This clonal heterogeneity can involve a number of mutations to key cell cycle control or tumour suppressor genes to facilitate the evasion of protective mechanisms within the cell (Leedham et al., 2008).

The association of GORD with the development of oesophageal adenocarcinoma is well established. Recent studies suggest that there is an initiator event linked with GORD which results in the transformation of a normal oesophageal squamous cell into a more malignant clone. Repeated episodes of reflux and chronic inflammation will then select for more resistant mutated clones which facilitates the progression of premalignant and malignant tissue (Maley, 2007).

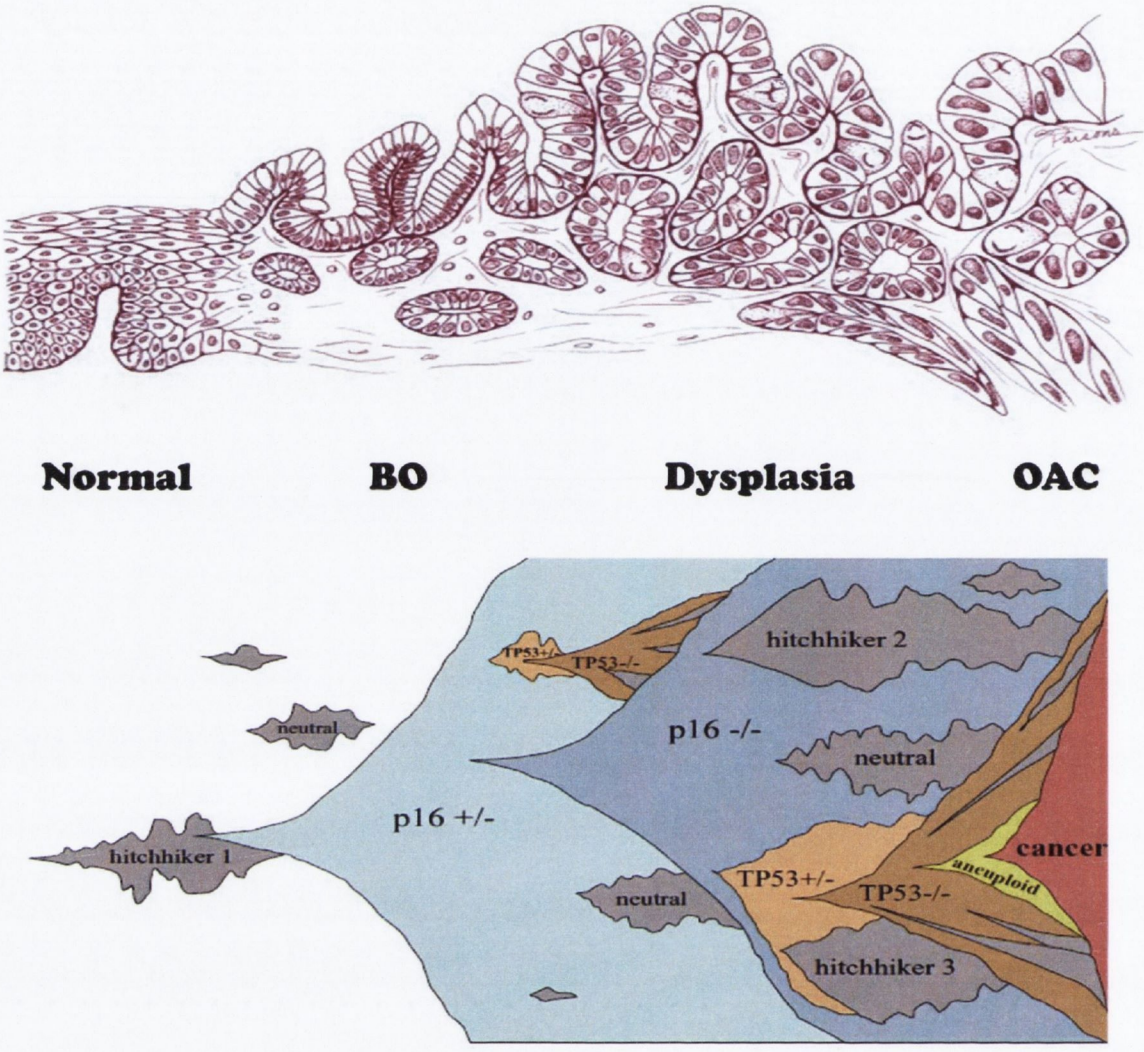


Figure 1.3 Non-linear clonal expansion and the oesophageal carcinogenic sequence. Clonal expansion occurs during the development of oesophageal adenocarcinoma in a non-linear fashion. Oesophageal cancer cells have acquired a number of genetic mutation conferring a significant competitive advantage on the cancer cells (modified from (Maley, 2007, Koppert et al., 2005)).

1.4 Gastro-oesophageal reflux disease

Chronic reflux causing inflammation and injury to the oesophagus caused by reflux of gastric and duodenal components is referred to as gastro-oesophageal reflux disease (GORD). GORD is a condition that afflicts an estimated 20-40% of the population of the Western world including the United States of American (Locke et al., 1997). This is highly significant as GORD has been demonstrated to be the most important risk factor, even more significant than age, associated with the development of BO (DeMeester et al.,

2001). GORD has been associated with BO since the 1970s (Spechler et al., 2010). An estimated 10% of individuals suffering from chronic reflux and consequent inflammation which presents as esophagitis will go on to develop BO (Jankowski et al., 1999).

In the 1990s, GORD was considered a requirement for the diagnosis of BO and there was even reluctance to perform endoscopies on patients presenting without GORD. This element of the definition of BO was deemed unreliable in the late 1990s and will be described in more detail in subsequent sections (Spechler et al., 2010). A contributing factor in the removal of GORD from the definition of BO was that it was discovered that long term GORD sufferers would often report a loss of symptoms of heart burn after a number of years. This is not due to a spontaneous clearance of the disease, but more likely a result of adaptation of the oesophageal mucosa to a more acid resistant phenotype in response to repeated exposure (DeMeester et al., 2001).

1.4.1 Oesophageal injury as a result of reflux

Gastric juices enter into the oesophagus as a consequence of a shortening of the lower oesophageal sphincter (LES) which results in a sudden reduction in pressure allowing gastric and duodenal contents to reflux into the oesophagus. Repeated incidents of reflux of gastric juices into the oesophagus can cause damage to the mucosal layer and more importantly to the LES muscle. Ultimately, sufficient damage is caused to the LES that leads to complete ineffectiveness in preventing entry of gastric juices to the oesophagus resulting in chronic reflux and the development of GORD (DeMeester et al., 2001). A further consequence of repeated exposure to reflux is the inability of the oesophagus to effectively clear gastric juice due to damage to underlying muscular layers. This decrease in the ability of the oesophagus to contract and expel the refluxate results in an increase in

the length of exposure time to harmful components of the refluxate (DeMeester et al., 2001).

Gastric contents introduced into the oesophagus during an episode of reflux result in a lowering of the normal pH. Other harmful components of reflux include conjugated and unconjugated bile acids, trypsin and lysolecithin (Vaezi and Richter, 1996). The introduction of these exogenous agents into the oesophagus results in epithelial damage which may expose normally protected stem cells in the basal layer of the oesophagus to acid and bile (Souza et al., 2008). The major components of refluxate that have been implicated in injury of oesophageal squamous mucosa are acid and bile salts (both conjugated and unconjugated) (Lagergren et al., 1999). It has been suggested that the squamous cells of the oesophagus adapt to the acidic environment caused by reflux of gastric juices. This adaptation is proposed to involve a number of phenotypic changes which results in decreased sensitivity to low pH (Kazumori et al., 2009). Chronic inflammation, induced by exposure to exogenous agents in the oesophagus, has also been demonstrated to induce persistent oxidative stress, which could also contribute to the development of BO (Chen and Yang, 2001).

1.4.2 The role of acid and bile salts in oesophageal carcinogenesis

Oesophageal injury occurs as a result of the exposure of healthy tissue to components of refluxate including acid which reduces oesophageal pH and duodenal elements such as bile salts. In patients who suffer from GORD, the concentration of bile acid in the refluxate correlates with the degree of injury to the oesophageal tissue (Kazumori et al., 2006). Multivariate analysis of factors associated with the development of Barrett's oesophagus identified exposure to bile acids as the most effective predictive factor (Vallbohmer et al., 2006). This would imply that bile acids are important drivers of the progression through the oesophageal adenocarcinoma sequence as a result of altered gene expression profiles.

The contribution of bile acids in the development of oesophageal adenocarcinoma is further emphasised by the fact that despite the development and availability of acid suppression therapies, metaplastic transformation and oesophageal adenocarcinoma continue to increase in prevalence. In the developed world, oesophageal adenocarcinoma is one of the most rapidly increasing cancer types (Vallbohmer et al., 2006).

It has been demonstrated that the more frequent and more severe incidences of reflux are, the greater the risk of development of oesophageal adenocarcinoma (Lagergren et al., 1999). Patients who reflux both gastric and duodenal components are found to have a higher prevalence of oesophagitis and BO (Vaezi and Richter, 1996). Acid reflux is a major factor in progression from benign oesophagitis to BO (Jankowski et al., 2000). The risk of development of OAC is increased 8 fold in people suffering from heart burn once a week. Suffering from reflux at night increases the risk of OAC development to 11 times that of the normal population (Lagergren et al., 1999). There is also an association between the degree of injury sustained and the concentration of bile acids that are present in the refluxate with the higher concentrations of bile acids corresponding with higher degrees of injury (Nehra et al., 1999).

Duodenal contents have been demonstrated to induce the expression of the intestinal related gene caudal type homeobox 2 (CDX2) in the oesophageal keratinocytes of rats which may initiate a transdifferentiation process resulting in the secretion of intestinal related mucins (Pera et al., 2007). Specifically, secondary bile acids stimulate the upregulation of CDX2 in normal oesophageal squamous cells which suggests a role for bile acids in the pathogenesis of BO (Hu et al., 2007). However, the effect of the bile acids in this study was observed at a high concentrations which may be above physiologically relevant levels and also may be toxic to the cells and as such requires further validation.

Exposure of oesophageal cells to acid has been demonstrated to increase the production of reactive oxygen species (ROS) and cause damage to DNA in the form of double strand breaks. It was originally hypothesised that there were potentially beneficial anti-proliferative effects resulting from acid exposure mediated by the p53 and Chk2 pathways, however it was demonstrated that these pathways may be activated indirectly due to double strand breaks in DNA (Zhang et al., 2009a). In this situation, a clone with a loss of p53 functionality would continue to survive regardless of the resulting DNA damage caused by acid, creating an unstable genetic environment. In this study, Zhang *et al* (2009) recommend acid suppression therapy as a chemotherapeutic strategy. However, Chen *et al* (2001) in an earlier review suggests that acid suppression therapy may contribute to mucosal damage caused by bile salts by regulating the pH environment (Chen and Yang, 2001). Interestingly, conjugation of bile acids results in a lowering of pKa so that conjugated bile acids can have synergistic damaging effects with acid caused lowering of the oesophageal pH. Unconjugated bile acids are then more potent at neutral pH, the normal state of the oesophageal environment (Guillem, 2005). Bile acids have been associated with a number of biological processes including cell proliferation, induction of DNA damage and the inhibition of apoptosis (Zhou et al., 2009).

Pulsatile exposure of BO to bile and acid has been demonstrated to increase the level of inflammation in the oesophagus through the induction of IL-1β. This effect is dependent on the pattern of exposure to acids and bile as continuous acid leads to a suppression of IL-1β expression (Fitzgerald et al., 2002). The importance of this relates to the conclusion derived from this study that expression of specific cytokines causes patterns of inflammation in BO. Exposure to bile and acid appears to induce and/or to suppress the expression of cytokines linked to inflammation. It has been demonstrated that bile and

acid exposure contributes to the regulation of inflammation, the proposed seventh hallmarks of cancer (Colotta et al., 2009). Interestingly, the contribution of bile acids to oesophageal disease pathogenesis is not entirely negative. The hydrophilic bile acid glycoursodeoxycholic acid has been demonstrated to be cytoprotective to oesophageal cells against the destructive effects of acid and bile through the inhibition of oxidative stress (Goldman et al., 2010).

1.4.3 Bile acids and Cell Death

Programmed cell death can occur through an apoptotic pathway or through autophagy. Evidence suggests that exposure to bile acids results in programmed cell death through the process of apoptosis (Dvorakova et al., 2005). Resistance to apoptosis is an important event in oesophageal carcinogenesis and will be discussed further in section 1.5.1.1. Apoptosis is a complex process involving a large number of molecules and control systems. There is a fine balance between the pro and anti-apoptotic molecules of the cell to ensure appropriate control of this process. Following exposure to a death signal, activated initiator proteins inhibit anti-apoptotic molecules such as B-cell lymphoma 2 (BCL2) and B-cell lymphoma 2 like 2 (BCL2L2 aka BCL-XL). This facilitates the release of cytochrome c from the mitochondria and subsequently the formation of the apoptosome. The apoptosome cleaves caspase 3 which results in programmed cell death through apoptosis (Degterev and Yuan, 2008). Apoptosis is characterised by the presence of DNA fragmentation, chromatin condensation, membrane blebbing, cell shrinkage and disassembly into membrane enclosed vesicles. The caspase family of proteases play an integral role in this process (Thornberry and Lazebnik, 1998). It has also been demonstrated that oesophageal cell lines can undergo programmed cell death through the process of autophagy. Autophagy has been shown to occur in response to external agents such as curcumin (O'Sullivan-Coyne et al., 2009).

Bile acids have been demonstrated to have a profound effect on normal cellular processes such as apoptosis and cell proliferation. The conjugated bile acid, glycochenodeoxycholic acid (GCDCA), increases the cell proliferation of a Barrett's cell line (BAR cells) but had no effect on a normal squamous cell line (Jaiswal et al., 2006). Resistance to apoptosis induced by DCA is a proposed mechanism adapted by BO tissue to gain a competitive advantage over normal squamous cells in an environment of chronic reflux (Dvorakova et al., 2005). A further important role for NF-κB in oesophageal malignancies was identified following the specific inhibition of the p65 subunit using a targeted siRNA. This inhibition of NF-κB resulted in an increase in apoptosis in an oesophageal cancer cell line following treatment with DCA, suggesting a role for NF-κB in the modulation of bile acid induced apoptosis in oesophageal cells *in vitro* (Burnat et al., 2010). These data suggest that bile acids play an integral role in the control and regulation of important cellular functions such as cell proliferation and apoptosis.

1.4.4 The development of cardiac or junctional mucosa

There is documented evidence of the presence of a columnar epithelium in the 'normal' population, specifically people who do not suffer from GORD or BO. This columnar mucosa is located at the gastroesophageal junction and is thought to function as a protective barrier against gastric juices. Columnar epithelium, also known as cardiac mucosa, does not produce pepsin or acid but is resistant to both. It is hypothesised that cardiac mucosa develops in response to brief exposure to gastric juices to protect oesophageal tissue that may otherwise be eroded if there was direct contact between oesophageal and gastric tissue. Cardiac mucosa has been demonstrated to express intestinal proteins such as CDX2 which illustrates that the cardiac mucosa may be a precursor to BO (Groisman et al., 2004). Positive immunohistochemical staining for a

number of intestinal markers were found in biopsies of oesophageal cardiac epithelium, providing further evidence that this type of mucosa may be an intermediate stage in the development of BO from normal squamous epithelium (Hahn et al., 2009).

1.5 Oesophageal adenocarcinoma

Oesophageal adenocarcinoma (OAC) is an aggressive disease with poor prognosis and limited treatment options if not detected early. The five year survival rates of this disease are reported to be less than 15% (Sundelof et al., 2002). Some of the factors associated with an increased risk of developing OAC include long standing reflux symptoms, night-time reflux, BO, obesity, using asthma medication and cigarette smoke. Studies have suggested that 5-10% of BO progresses to dysplasia with an estimated 1% of these patients developing OAC (DeMeester et al., 2001).

Dysplasia refers to neoplastic epithelium that is confined to the basement membrane of the oesophagus and as such is considered a precancerous lesion with an increased risk of progression. Dysplasia is identified and graded based on the presence of a number of changes. These changes included glandular distortion and crowding, alterations in the size and shape of the nucleus, increased nuclei to cytoplasmic ratio, hyperchromatism and an increased number of abnormal mitoses (Flejou, 2005). The grade of dysplasia, low or high, is determined by the degree of abnormalities present. A higher grade of dysplasia is associated with an increased risk of adenocarcinoma development. A correct diagnosis of the grade of dysplasia present is therefore an important factor in the surveillance strategy designed for the patient (Geboes and Van Eyken, 2000).

Progression to adenocarcinoma is defined as the invasion of abnormal cells to mucosa or submucosa layers of oesophageal epithelium. Oesophageal adenocarcinoma has a number

of defined stages, with the stage of cancer present at diagnosis determining the treatment course that is undertaken. Stage I involves the spreading of abnormal cells beyond the innermost layer of the oesophagus into the wall of the oesophagus. Patients presenting with stage I cancer undergo endoscopic mucosal resection or endoscopic submucosal dissection. The further of spreading of the abnormal cells to the oesophageal muscle or outer wall of the oesophagus is designated stage IIA. Stage IIB involves the spreading of the cancer to any of the first three layers of the oesophagus or to nearby lymph nodes. The recommended treatment for this cancer stage is oesophagectomy. Stage III cancer is identified by the presence of cancer in the outer wall of the oesophagus and may have spread to tissues or lymph nodes located near the oesophagus. Stage III cancer is also treated with oesophagectomy, often in conjunction with chemoradiotherapy. The final stage of oesophageal cancer, stage IV, is divided into two subsections depending on where the cancer has spread. Stage IVA classifies cancers with spreading to nearby or distant lymph nodes. Cases where the cancer has spread to distant lymph nodes and/or organs in other parts of the body are classified as stage IVB cancers. Unfortunately, patient presenting with stage IV metastatic disease have a very poor prognosis with the only treatment option offered being palliative care (Diederich, 2007, Spechler et al., 2010).

1.5.1 Underlying genetics of the oesophageal carcinogenesis

Cancer cells acquire a number of phenotypic alterations during the course of their development which confers upon them a selective advantage over normal cells. These traits were characterised by Hanahan and Weinberg in 2000 (Hanahan and Weinberg, 2000). Six hallmarks of cancer were described: evasion of apoptosis, sustained angiogenesis, limitless replicative potential, self-sufficiency in growth signals, insensitivity to growth inhibition and tissue invasion & metastasis. Inflammation has recently been proposed as the seventh hallmark of cancer (Colotta et al., 2009) (Figure 1.4). Developing

these hallmarks of cancer requires normal cells to undergo a number of genetic changes. In the case of oesophageal adenocarcinogenesis, a number of these genetic alterations have been documented.

1.5.1.1 Acquiring the hallmarks of cancer and oesophageal adenocarcinoma

DNA damage has been demonstrated in the form of double strand breaks (DSB) in oesophageal cell lines caused by reactive oxygen species (ROS) resulting from acid exposure (Zhang et al., 2009a). DNA damage results in the accumulation of p53 protein which stalls the cell cycle at G1, allowing genetic damage to be repaired. If repair of genetic damage fails, p53 promotes apoptosis. Cells with loss of expression of p53 can evade apoptosis which confers a selective advantage. Loss of p53 also facilitates increased genetic instability, which allows for an accumulation of genetic mutations (Maley, 2007).

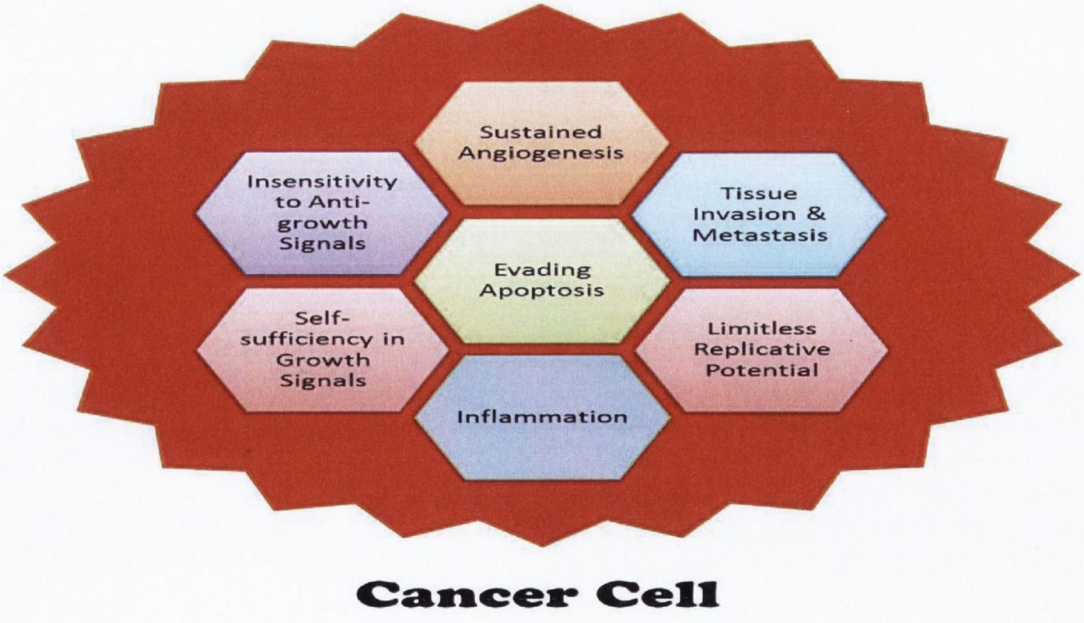


Figure 1.4: Hallmarks of cancer. The original hallmarks of cancer as proposed by Hanahan and Weinberg (2000) are sustained angiogenesis, tissue invasion & metastasis, limitless replicative potential, evading apoptosis, self-sufficiency in growth signals and insensitivity growth signals. Colotta *et al* (2009) proposed that inflammation is the seventh hallmark of cancer (Colotta et al., 2009, Hanahan and Weinberg, 2000).

It has been demonstrated that loss of chromosome 17p, the location of the p53 gene, is a frequent event in oesophageal adenocarcinogenesis. A number of papers have reported the loss of p53 to varying degree from each stage of the carcinogenic sequence. There is a general consensus that loss of p53 does occur in the development of oesophageal adenocarcinoma, however further work is required to give a comprehensive view of the levels of p53 at the different stages of the carcinogenic sequence. Current reports indicate that loss of p53 allele has been detected in 79% of adenocarcinoma and appears to be an early event in the carcinogenic sequence as 30-66% of high grade dysplasia and up to 60% of metaplasia have been found to have mutations in p53 (Prevo et al., 1999, Schneider et al., 1996, Jenkins et al., 2002). Loss of p53 expression in BO patients is associated with a 16 fold increase in the risk of developing adenocarcinoma (Reid et al., 2001). Identical mutations in p53 have been detected in both dysplastic tissue and adjacent OAC tissue, suggesting that p53 mutation is an early event during disease development (Gleeson et al., 1998). Also, the negative regulator of p53, transformed mouse 3T3 cell double minute 2 (MDM2; mouse homolog), is overexpression in OAC leading to an inactivation of p53 (Jenkins et al., 2002). Evasion of apoptosis by loss of p53 activity is an important event in oesophageal adenocarcinogenesis.

Resistance to apoptosis induced by DCA has been demonstrated in *ex vivo* Barrett's samples compared to normal and colonic tissue (Dvorakova et al., 2005). Further evidence of evasion of apoptosis during adenocarcinogenesis is the reported switch to a more apoptotic resistant genotype that has been illustrated during progression from intestinal metaplasia to adenocarcinoma. Increased expression of the anti-apoptotic protein BCL-XL and decreased expression of the proapoptotic protein BAX was demonstrated in adenocarcinoma biopsies compared to intestinal metaplasia biopsies using immunohistochemistry (van der Woude et al., 2002) (Figure 1.5).

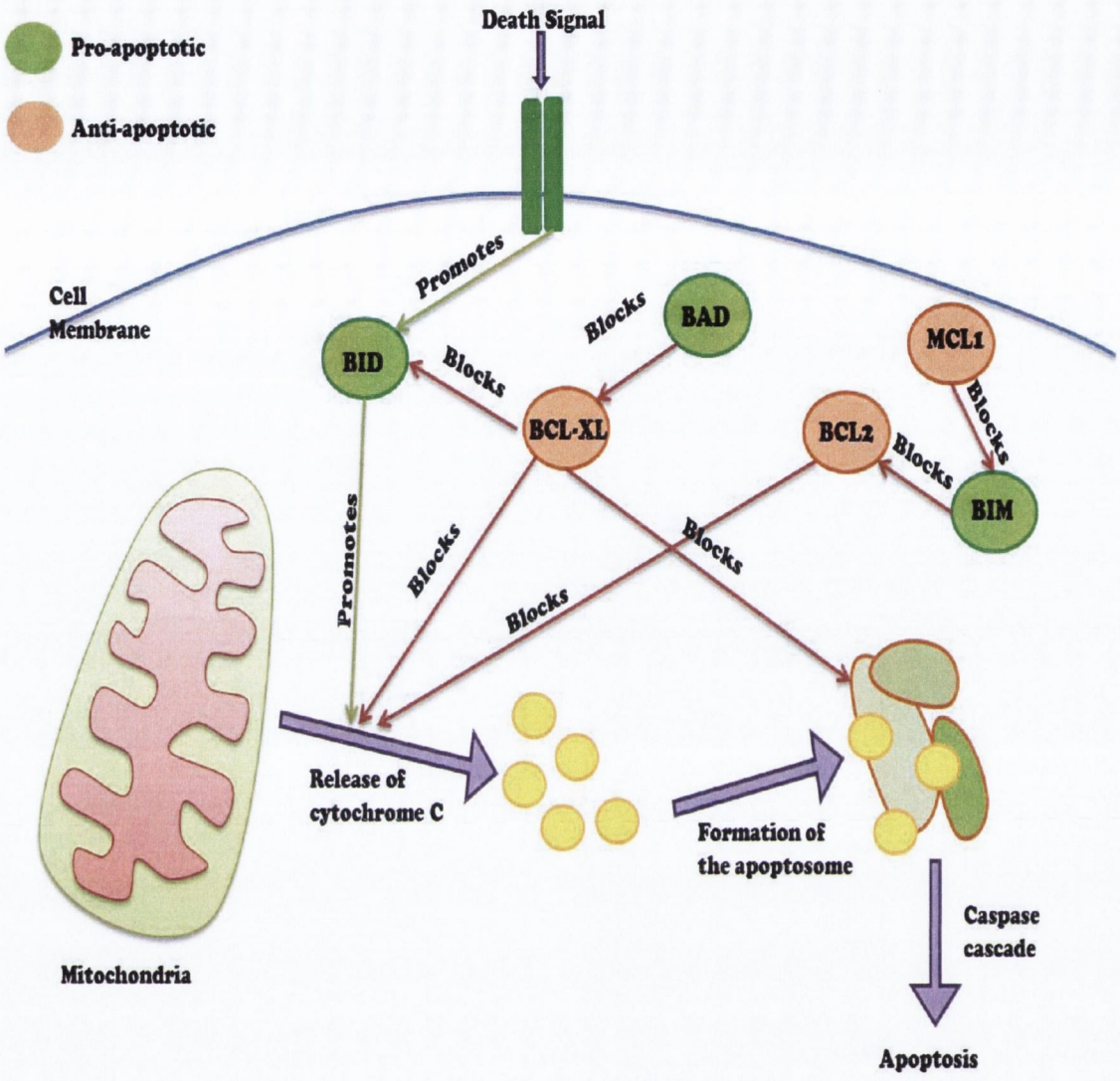


Figure 1.5: Apoptosis pathway. Extract of the apoptosis pathway highlighting some of the molecules that have been implicated in oesophageal adenocarcinoma development. BCL-XL is an anti-apoptotic protein that inhibits the release of cytochrome C and the formation of the apoptosome. MCL1, also an anti-apoptotic protein, indirectly blocks the release of cytochrome C from the mitochondria through the inhibition of the BCL2 inhibitor BIM.

Cyclooxygenase-2 (COX2) is involved in promoting resistance to apoptosis (Souza et al., 2000) and has been implemented in oesophageal adenocarcinogenesis. COX2 may have a role in the development of oesophageal adenocarcinoma as the expression levels of COX2 increase during progression through the carcinogenic sequence. The inducibility of COX2 in response to bile salt and acid has also been demonstrated, further evidence for its role in oesophageal adenocarcinogenesis (Shirvani et al., 2000).

Vascular endothelial growth factor (VEGF) is known to contribute to angiogenesis and is frequently used as a marker for tumour vascularisation (Kleespies et al., 2004). The angiogenic phenotype of each stage of the oesophageal carcinogenic sequence was determined by assessing the expression level of VEGF and also by examination of the neovascularisation coefficient. The neovascularisation coefficient is a measurement of the relationship between newly formed and pre-existing capillaries. It was discovered that VEGF expression and the neovascularisation coefficient both increase significantly during progression from BO to high grade dysplasia (HGD) and again from HGD to OAC (Mobius et al., 2003). VEGF has also been shown to be inducible by the deoxycholic acid (Burnat et al., 2007). This evidence indicates sustained angiogenesis during the oesophageal carcinogenic sequence.

Telomeres are long stretches of non-coding DNA that maintain chromosomal integrity and provide genomic instability. Due to the limitation of cellular replication machinery to replicate to the very end of each chromosome, each cycle of replication results in shortening of the chromosome and loss of a portion of telomeres. A critical level is reached where the chromosome no longer possesses a sufficient number of telomeres to protect the chromosome and the cell becomes senescent. This limited replicative potential is generally overcome by cancer cells by the overexpression of the telomerase enzyme. This enzyme maintains the telomere length on the chromosome, thus allowing for limitless replicative potential (Shay and Bacchetti, 1997). Telomerase expression is greatly increased in all stages of the oesophageal carcinogenic sequence compared to normal tissue. There is also increased telomerase activity in both adenocarcinoma and dysplasia tissues compared to intestinal metaplasia tissue. These observations suggest that increased telomerase activity occurs early in the carcinogenic sequence and continues to rise with progression through the sequence (Lord et al., 2000).

Telomerase activity is greatly increased in an oesophageal adenocarcinoma cell line, BIC1, compared to normal gastric/intestinal cells. Cell proliferation and reduced colony numbers were observed in BIC1 cells that had been exposed to a telomerase inhibitor, indicating a role for telomerase in facilitating limitless replicative potential in oesophageal adenocarcinoma cells (Shammas et al., 2004). These studies together illustrate that telomerase expression increases with progression through the oesophageal carcinogenic sequence and that the telomerase activity facilitates limitless replicative potential.

1.6 Microarray

The development of revolutionary technologies in the field of transcriptomic research in the last twenty years has laid the foundation for unprecedented advances in investigations of the underlying molecular mechanisms of various diseases (Altshuler et al., 2008). When the completed human genome sequence was incorporated into these newly developed technologies, analysing the entire genome in the context of a single disease became a reality (Venter et al., 2001, Lander et al., 2001). This reality has since been realised utilising technologies such as microarray analysis and more recently with the advent of Next Generation Sequencing (Morozova et al., 2009). A leading example of the potential of these technologies is in the case of breast cancer. As a direct result of research carried out using microarray technology, a paradigm shift has occurred in the manner in which this disease is perceived. Microarray gene expression profiles of breast cancer tissue have predicted new classes of tumours; identified possible aggression levels of individual tumours and indicated the possibility of identifying tumours most likely to respond to treatment (Weigelt et al., 2010). The unprecedented success of microarray technology integrated into breast cancer research signifies the potential advances that may be accomplished in other disease areas using a similar approach. Microarray technology does

have limitations and draw backs but is an important and powerful tool in unravelling the underlying mechanisms of various diseases.

1.6.1 Oligonucleotide microarray

There are two types of gene expression microarray systems available which are cDNA microarrays and oligonucleotide microarrays. The microarrays relevant to this study are all oligonucleotide arrays produced by Affymetrix. These arrays are comprised of a string of nucleotides attached to a solid surface which act as probes to bind to complementary sequences of the target genes for the quantification of expression levels. The advent of this technology is based on significant advances in the production of solid chips that can accommodate hundreds of thousands of targets in defined areas of the base. Advances in detection methods using laser confocal fluorescence scanning also contribution to the development of microarray technology (Lipshutz et al., 1999). In the case of the newer version of these arrays, the entire genome can be targeted using a single chip (GeneChip Human Genome U133 Plus 2.0 Array) (Affymetrix, 2011). Microarray technology exploits the complementarity of nucleotide base pairs to detect the expression levels of messenger RNA (mRNA) in a sample. Oligonucleotide arrays contain probes affixed to a solid surface that will bind specifically to labelled targets allowing for the quantification of the expression levels of each gene of interest in experimental sample (Figure 1.6). The mRNA is extracted from the sample of interest, cells or tissues and reverse transcribed into cDNA. This cDNA is labelled during a transcription step resulting in the generation of fluorescently labelled cRNA. The cRNA is incubated with the oligonucleotide array where complementary sequences hybridise. Following a series of washing steps, the array is scanned and the expression levels of genes from the original sample is determined following quantification of the level of fluorescent signalling. Software packages such as GeneSpring™ facilitate the analysis of the raw data.

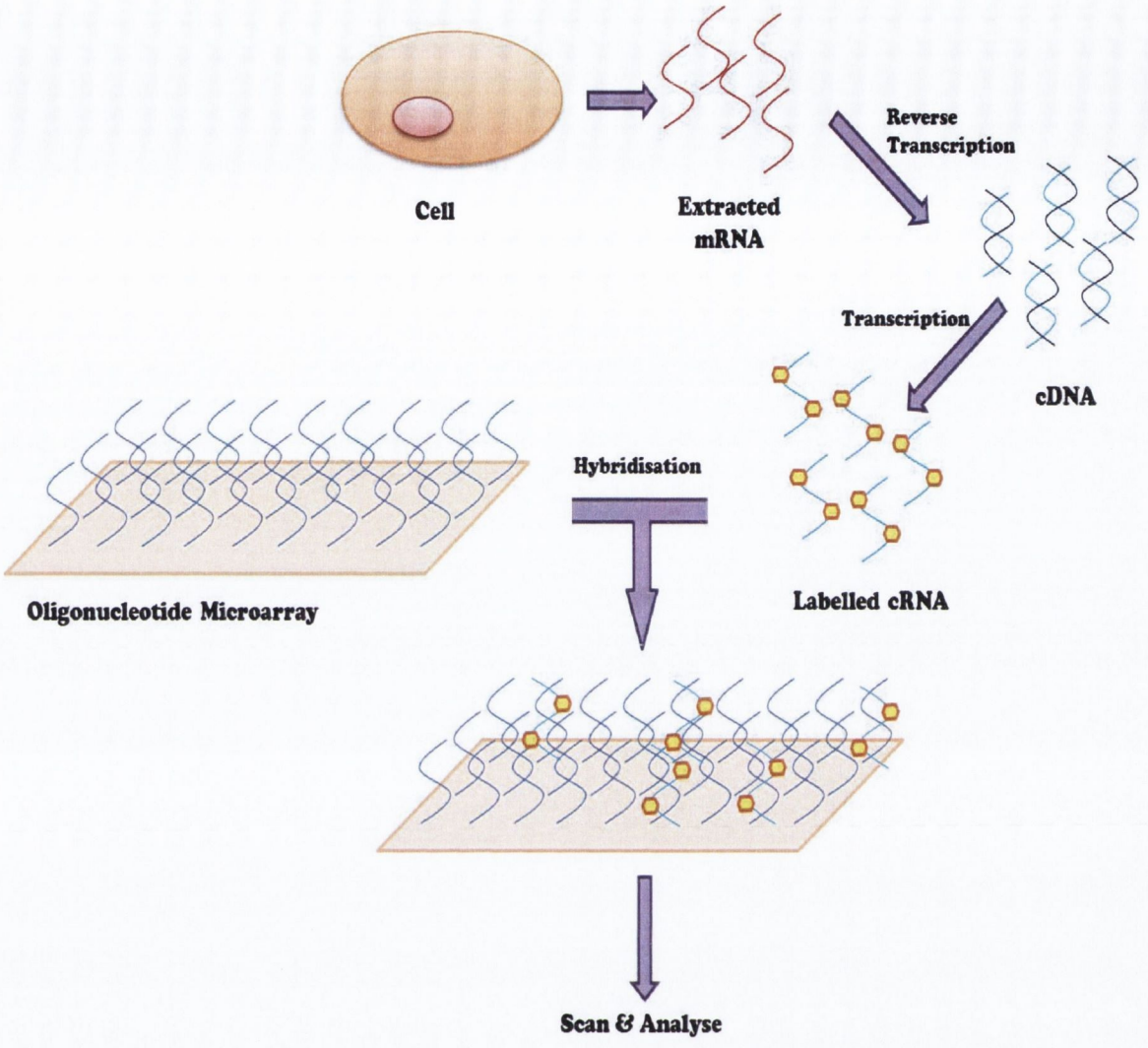


Figure 1.6: Oligonucleotide microarray. The mRNA is extracted from the cell/tissue of interest and reverse transcribed into cDNA. The cDNA is labelled and transcribed to give a pool of labelled cRNA. The cRNA hybridises to complementary oligonucleotides affixed to a solid surface. The array is scanned using a laser confocal scanning technique generating fluorescent readings corresponding to the levels of expression of the genes in the original sample. Software packages such as GeneSpring™ 7.0 are available to facilitate analysis of the gene expression microarray data.

Oligonucleotide arrays have a high level of specificity and sensitivity as a result of the selectivity of detection provided by the complementarity of strands of nucleic acids (Duggan et al., 1999). An advantage associated with oligonucleotide arrays is that the synthesis of the probes is based on the sequence of the target. Probes can be designed to complement the most unique sequence of the targets to give added specificity to the system. This approach also reduces cross reactivity with family members and also can

facilitate the detection of splice variants (Schulze and Downward, 2001). As a result of advances in the miniaturisation of microarrays, the expression of a large number of genes can be assessed simultaneously without the need to estimate important genes that may be involved in the processes under examination based on current knowledge (Lockhart and Winzeler, 2000). Oligonucleotide arrays contain a number of control features to ensure the production of accurate results. Multiple probes targeting non-overlapping sequences of the target gene are included to allow hybridisation to different regions of the same target sequence. This redundancy feature improves accuracy allowing for a reduction in the signal to noise ratio and reduces false positives. Specificity of hybridisation is controlled for by the inclusion of probes with a mismatched base in the central region of the probe. This facilitates the detection of non-specific binding to probes (Lipshutz et al., 1999).

Oligonucleotide microarrays are associated with a degree of variability and the use of replicates is encouraged. This process is expensive and not routinely available in many laboratories, therefore rigorous validation and verification of results in recommended, especially if replication opportunities are limited. Functional studies are also required to determine the specific contribution of each gene to the observed phenotype to confirm that the detected expression levels are causative as opposed to a consequence of upstream alteration (Schulze and Downward, 2001).

1.6.2 Gene expression signatures

The varied response to treatment of patients with apparently morphologically similar cancers is thought to occur as a result of an underlying heterogeneity which has driven the development of more accurate classification systems (Golub et al., 1999). Microarray studies in breast cancer research have identified novel subclasses of the disease which will contribute to a greater understanding of the prognosis and predicted response to treatment

between individual tumours (Weigelt et al., 2010). This demonstrates that a more accurate classification method could contribute to more successful application of currently available therapies. The capacity of microarray technology to function as a tool in the classification of cancers was explored in the case of human acute leukaemia. This study demonstrates that gene expression signatures derived from microarray experiments can successfully establish classes of cancer with similar transcriptomic profiles and can also classify unknown tumours using a predictor model developed from the expression profiles of cancers from previously described classes (Golub et al., 1999).

In the case of oesophageal malignancies, gene expression profiles in conjunction with various bioinformatic tools have been shown to have the ability to differentiate between healthy and diseased samples. Unsupervised hierarchical clustering of oesophageal tissue samples of various stages of disease successfully segregated these samples into cancer subtypes indicating the potential of this technique as an objective diagnostic tool. This tool may be incorporated into the current diagnosis strategy involving histopathological methods to improve the accuracy of diagnosis (Selaru et al., 2002).

Further evidence demonstrating the versatility and power of microarray technology comes from studies suggesting that gene expression signatures can determine classes of functional related genes, known as modules that may contribute to the development of disease. Modules that have been identified in this manner as having aberrant expression profiles at different stages of the carcinogenic sequence include lipid metabolism which was found to be active in intestinal metaplasia but inactive in adenocarcinoma samples whereas a cytokine related module is inactive in normal and oesophagitis samples (Gomes et al., 2005). Analysis of gene expression signatures using modules has the potential to develop multiple research directions in relation to diagnostic, prognostic and therapeutic studies as

demonstrated in an integrated analysis of 1,975 published microarrays across 22 different tumour samples. This study identified modules that were activated or deactivated in a tumour specific manner but also revealed a number of modules that were responsive in a similar manner across widespread tumour types suggesting common tumour progression mechanisms (Segal et al., 2004). This global analysis of gene expression profiles allows for the integration of existing biological knowledge. In yeast, analysis of gene expression signatures has been used in an attempt to identify modules of coregulated genes, regulators of expression and conditions under which regulation occurs (Segal et al., 2003). While limitations have been identified in this system such as false positives and the failed identification of related regulatory relationships, this procedure highlights a further application of gene expression profiling.

1.7 RNA interference

Since it was first described just over a decade ago, RNA interference (RNAi) has evolved significantly from causing a worm to twitch to where currently, RNAi is being developed as a novel therapeutic strategy that could revolutionise how diseases are treated. The specific inhibition of target genes using RNAi molecules is a well-established research tool to identify gene function and is also a highly promising therapeutic strategy to inhibit the expression of disease causing genes. RNAi, if successfully developed as a functional treatment strategy, could be utilised to sensitise drug resistant tumours, induce apoptosis in immortal cells, knockout the expression of a disease causing genes and many other applications.

Andrew Fire and Craig Mello received the Nobel Prize in Physiology or Medicine in 2006 for their ground-breaking study determining specific genetic interference by double-stranded RNA (dsRNA) in *Caenorhabditis elegans.* This study, for the first time,

demonstrated that dsRNA injection in *C. elegans* using sequence homology to a target gene could inhibit the expression of that gene to such a level as to be characterised phenotypically. The observed phenotype was extremely specific and mirrored exactly the previously determined phenotype expected from a loss of function mutation. Not only did this study discover this remarkable function of dsRNA, it also accurately hypothesised that the mechanism invoked was post-transcriptional causing a significant reduction in the mRNA levels of the target gene (Fire et al., 1998). The theory was given additional support as the DNA sequences of the target genes remained unaltered after dsRNA mediated expression interference. It was also discovered that the cytoplasmic accumulation of the mRNA levels of the target gene was almost completely eliminated. These data indicated that the endogenous target mRNA was degraded before being translated into protein and that this process was triggered by the introduction of a dsRNA homologous sequence (Montgomery et al., 1998).

These innovative studies resulted in a paradigm shift relating to one of the most basic cellular processes, the translation of mRNA into a functional protein (Figure 1.7). This work has demonstrated that a conserved mechanism exists in plants, fungi and animals capable of unravelling dsRNA and using the complementarity of the resultant strands to target the degradation of mRNA (Mello and Conte, 2004). This mechanism in humans was successfully identified using small inhibiting/interfering RNA (siRNA) molecules targeting firefly reporter genes. The siRNA and a reporter plasmid were cotransfected into human cell lines resulting in the lack of expression of the reporter genes as a consequence of the siRNA targeted inhibition (Elbashir et al., 2001).

The process by which RNAi functions in mammalian cells has since been elucidated and is illustrated in Figure 1.8. Long dsRNA is processed by the RNAse III enzyme Dicer

creating a pool of siRNA molecules. The length of the naturally processed siRNA (21/22 nucleotides) corresponds to distance from the binding domain of the DICER enzyme to the active site of the RNAse III domains. DICER binds specifically to the dsRNA 5' phosphate with a two-nucleotide 3' overhang. Processing of the siRNA is facilitated as DICER binds to the TAR RNA-binding protein (TRBP/TARBP2P) or protein kinase R (PKR)-activating protein (PACT). This complex, including the double stranded siRNA, interacts with the RNA-induced silencing complex (RISC). The double stranded siRNA is unwound with incorporation of the guide strand into the complex. RISC can then target complementary mRNA for degradation. A single guide strand can direct the degradation of a number of the target mRNA molecules (Phalon et al., 2010).

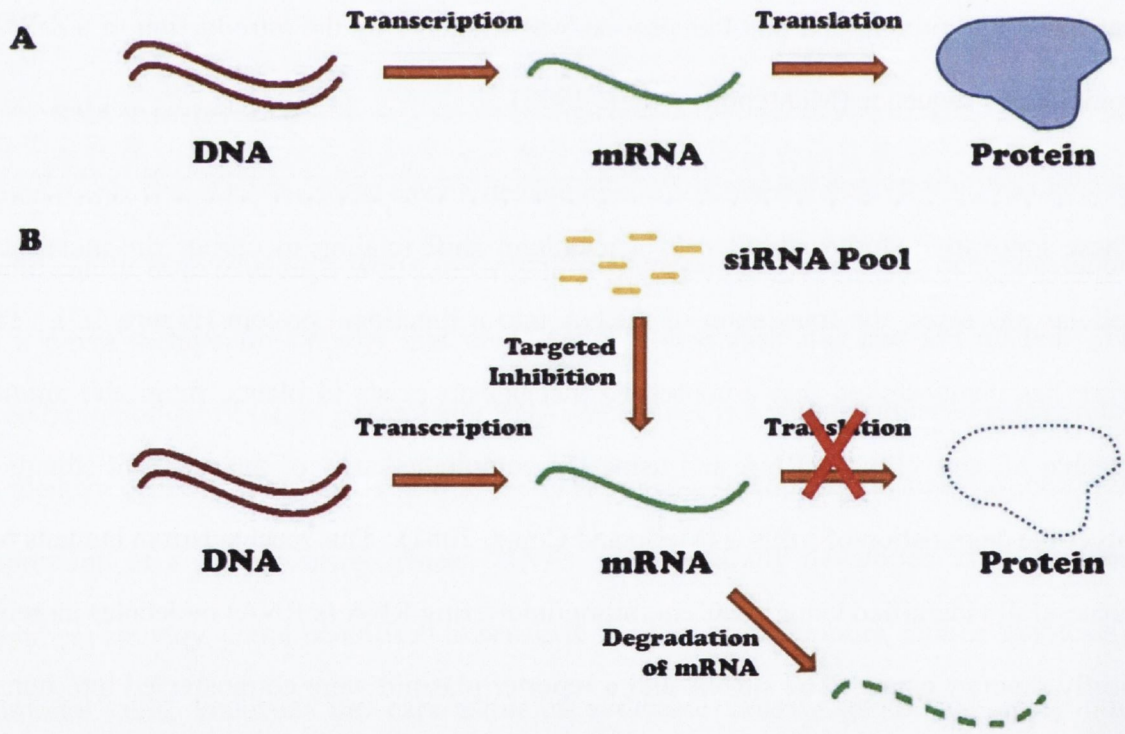


Figure 1.7: The discovery of RNAi resulted in a paradigm shift regarding the proposed pathway of gene expression. (A) Before the discovery of RNAi in 1998, it was generally accepted that messenger RNA was translated into protein without little or no post-transcriptional control. Expression levels were regulated at a DNA or protein level only. (B) The breakthrough discovery of RNAi revealed an important post-transcriptional regulation of gene expression which resulted in the degradation of mRNA by specific targeted inhibition, rendering loss of translation of protein.

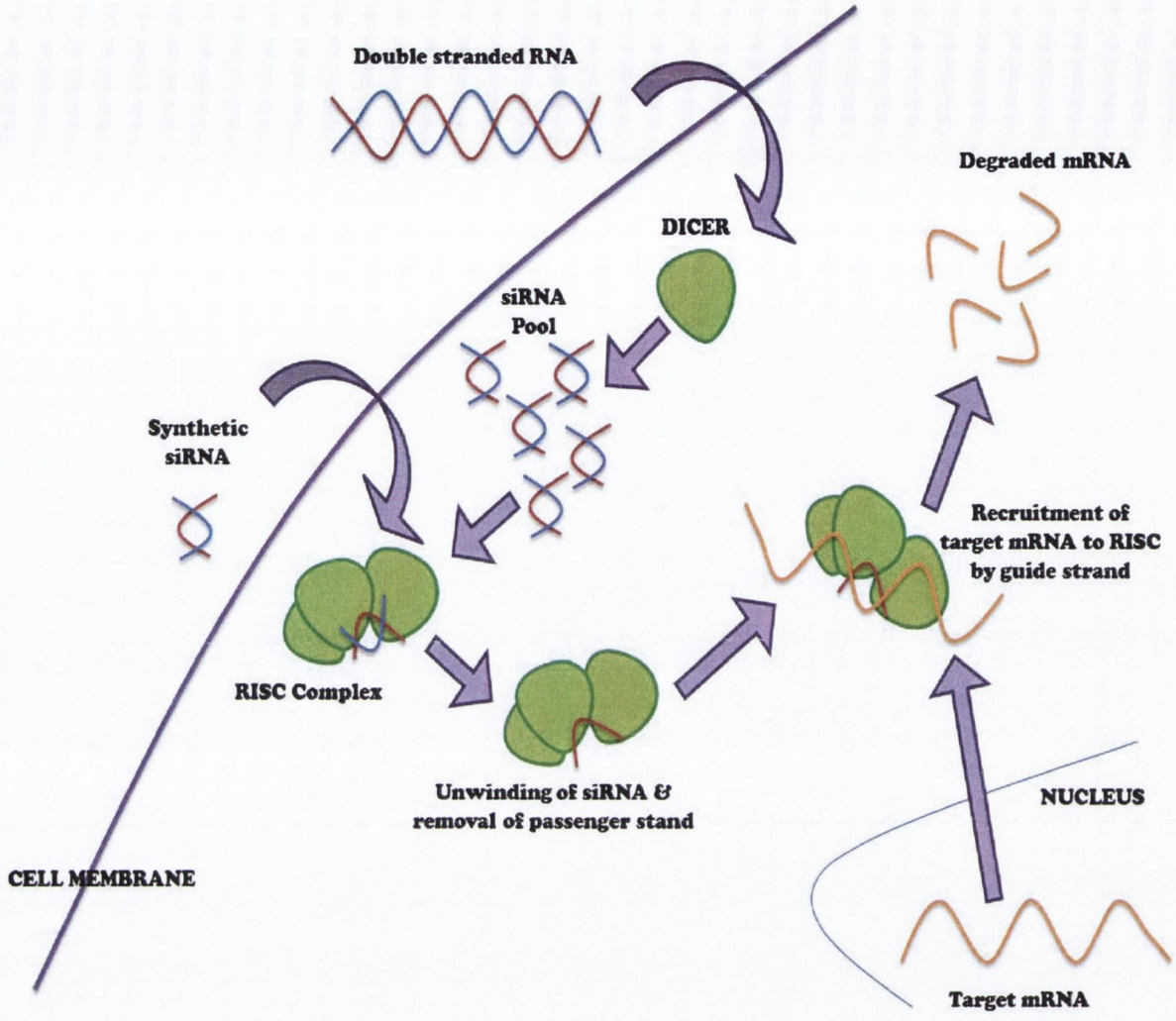


Figure 1.8: Specific inhibition of targeted mRNA using siRNA. Double stranded siRNA is fragmented in smaller siRNA duplexes approximately 21 nucleotides in length by DICER. Synthetic siRNA is manufactured to mimic this process. The siRNA molecules are recruited to the RISC complex where the double strands are unwound and the passenger strand is expelled from the complex. The guide strands binds the target mRNA facilitating the cleaving of the target mRNA and subsequent degradation of the mRNA.

Synthetic pools of siRNA duplexes are created to mimic the process that occurs naturally in the cell. The manufactured siRNA molecules are equal in length to the naturally occurring pools and as such do not require processing through DICER. Commercially produced siRNA pools utilise sophisticated bioinformatics design processes and algorithms to maximise specificity, efficiency and potency. In order to avoid saturating the different elements of the RNAi processing mechanism with non-functional siRNA molecules, only the most efficient siRNAs are selected for inclusion in the pools. Non-overlapping siRNA

molecules are also chosen to allow for maximum efficiency. The pooling of synthetic siRNA molecules also reduces the influence of off-target effects as lower concentrations of each individual molecule are required (Dharmacon, 2011).

1.7.1 High throughput & high content siRNA screens

Advances in the fields of automated fluorescent microscopy and improvements in liquid handling robotic systems has resulted in wider application of high throughput/high content (HT/HC) siRNA screens. The availability of libraries of siRNA pools targeting families of molecules has also contributed to the publication of a growing number of studies involving siRNA screens. Dharmacon currently produce a number of SMART-pool siRNA libraries including those targeting the whole genome, the druggable genome, G-protein coupled receptors (GPCRs) and kinases family members.

HT/HC screening has been used to identify gene targets capable of sensitising a non-small-cell lung cancer line to sub-lethal concentrations of paclitaxel, a drug routinely used in the treatment of this disease. The study implemented a whole genome screen using a one-well/one-gene approach. The endpoint assay implemented in this study was a well-established luminescent based cell viability assay. This type of study where a chemotherapeutic drug is used in conjunction with siRNA inhibition to sensitise cells is known as a synthetic lethal screen. This study successfully identified several targets that reduced cell viability significantly in the presence of the drug (Whitehurst et al., 2007). A similar synthetic lethal approached was successfully utilised to identify gene targets whose inhibition sensitised a breast cancer cell line to inhibitors of poly (ADP-ribose)-polymerase-1 (PARP). This study utilised a library targeting kinases and concluded that novel therapeutic targets can potentially be identified using synthetic lethal siRNA screens in conjunction with chemical inhibitors (Turner et al., 2008). The kinase subset of the

human genome was again systematically targeted, along with the family of phosphatases, in an siRNA screen to identify anti-apoptotic survival pathways. This study demonstrates another function of siRNA high throughput screening as several new survival kinases were identified, as was a novel role for phosphatases as negative regulators of apoptosis (MacKeigan et al., 2005).

This brief description of the manner by which siRNA screens have been previously utilised highlights some of the potential applications of siRNA screens. Current work developing dynamic screening systems and improvements in data mining processes will increase the potential of this powerful tool that was first described 12 years ago in a twitching microscopic worm.

1.7.2 RNAi and oesophageal cancer

RNAi has been successfully utilised to assess the functional relevance of a number of genes in oesophageal cancer. Targeted inhibition using RNAi has demonstrated that loss of function of β-catenin results in a reduction of cyclin D1, colony formation and growth inhibition of an oesophageal cell line. *In vivo*, specific inhibition of β-catenin resulted in a substantial decrease in the rate of tumour growth in nude mice (Wang et al., 2009). Barrett's epithelium has been demonstrated as more resistant to apoptosis induced by UV-B irradiation causing DNA damage than normal squamous cells. This resistance was overcome following the specific inhibition of NF-κB, and more potently, BCL2 using a targeting siRNA molecule (Hormi-Carver and Souza, 2009). These studies demonstrate that RNAi is a powerful research tool in the field of oesophageal cancer research and will be an important feature in the development of future therapies.

1.8 Aims

The aims of this study are:

1. to utilise an understanding of the functional genomics of genes induced by bile acids to identify new targets for oesophageal cancer therapy.

2. to generate new knowledge regarding the mechanisms whereby oesophageal cells become resistant to DCA-induced apoptosis.

3. to use a high throughput/high content siRNA screening approach to dissect mechanisms involved in oesophageal carcinogenesis.

4. to determine mechanisms involved in the regulation of the intestinal phenotype in oesophageal carcinogenesis.

Chapter 2

Materials & Methods

2 Materials & Methods

2.1 Cell Culture

2.1.1 Maintenance of cell lines

Cell lines were maintained as described in Table 2.1. All media was stored at 4°C and heated to 37°C before use. Stock solutions of penicillin-streptomycin-l-glutamine (PSG) (Sigma, Poole, Dorset, UK), foetal calf serum (FCS) (Gibco/Biosciences, Dublin, Ireland), BEBM supplement kits (Lonza, Berkshire, England) and trypsin-EDTA (Gibco/Biosciences, Dublin, Ireland) were aliquoted and stored at -20°C. FCS was heat inactivated at 56°C for 60 minutes to inactivate complement before aliquoting and storage. Cells were fed every 48 hours and were split approximately every four days depending on seeding concentration and requirement of experimental use.

2.1.2 Subculturing of cells

All cell lines used were adherent and required treatment with trypsin-EDTA to facilitate subculture of the cell lines. The trypsin-EDTA was purchased at 10 X concentration and diluted to 1 X concentration for use in Hank's balanced salt solution (HBSS) (Gibco/ Biosciences, Dublin, Ireland). For splitting, complete media was removed from the cells. Cells were washed briefly with HBSS which was replaced with trypsin-EDTA in HBSS. Cells were trypsinised at 37°C for approximately 5 minutes. The cells in suspension were pelleted by centrifugation for 5 minutes at 1,500rpm. The pellet was resuspended in complete media and cells were reseeded at an appropriate concentration depending on the experimental procedure requirements.

2.1.3 Long term storage and resuscitation

Stocks of cells were maintained by cryopreservation. Cells were frozen in FCS with 10% dimethyl sulfoxide (DMSO) stored at -80°C. For long term storage, cells were transferred

to liquid nitrogen. Cells were resuscitated by rapid defrosting and resuspension in warmed complete media. These cells were allowed to adhere to tissue culture flasks overnight. The media on these cells was then replaced to remove any DMSO which acts as cryoprotective agent.

Table 2.1: Cell lines, media and conditions used in this study

	Origin of Cells	Media	Supplements	Supplier	Source
Het-1A	Normal Oesophagus	BEBM	BEBM Supplement Kit	Lonza	ATCC
QhTRT	Non-dysplastic Barrett's Oesophagus	BEBM	BEBM Supplement Kit; 5% FCS	Lonza; Gibco/ Biosciences	Gift: Dr. Rabinovitch
GOhTRT	Dysplastic Oesophagus	BEBM	BEBM Supplement Kit; 5% FCS	Lonza; Gibco/ Biosciences	Gift: Dr. Rabinovitch
SKGT4	Oesophageal Adenocarcinoma	RPMI 1640	10% FCS; PSG*	Gibco/ Biosciences; Sigma Aldrich	Gift: Dr. Schrump
AGS	Gastric Adenocarcinoma	RPMI 1640	10% FCS; PSG*	Gibco/ Biosciences; Sigma Aldrich	ATCC
Caco2	Colorectal Adenocarcinoma	EMEM	20% FCS	Gibco/ Biosciences	ATCC
OE33	Adenocarcinoma of lower oesophagus	RPMI 1640	10% FCS; PSG*	Gibco/ Biosciences; Sigma Aldrich	ECACC
HCT116	Colon Carcinoma	McCoy's 5A Medium Modified	10% FCS	Sigma Aldrich	ATCC

*PSG: 100units/ml penicillin + 100µg/ml streptomycin + 2mM L-glutamine
All cell lines were maintained at 37°C in a 5% CO_2 humidified atmosphere.

2.1.4 Cell counting

Experimental procedure typically required an exact number of cells to be seeded into each well to ensure consistency between experiments. Cell numbers were estimated using a haemocytometer (Figure 2.1). Following placement of a cover slip securely over the haemocytometer grid, 8µl of cell suspension was pipetted onto the grid. Cells that were

located in the four sets of 16 squares highlighted by red circles in Figure 2.1 were counted. This number was divided by 4 to an average per set of 16 squares. The resulting value was multiplied by ten to estimate the number of cells per microliter. A solution with the appropriate number of cells was prepared using this estimate of cell number.

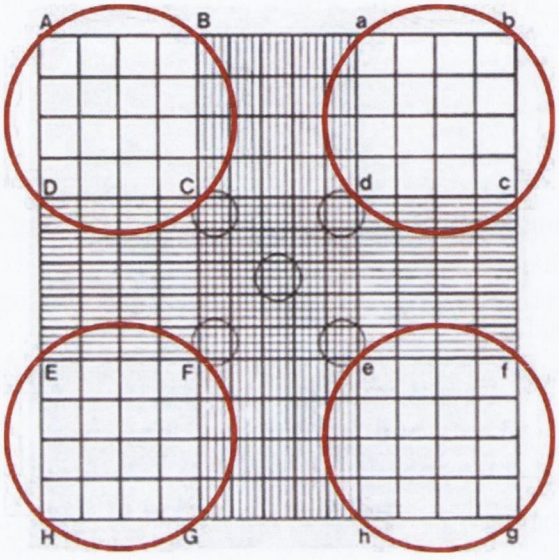


Figure 2.1: Image of a haemocytometer grid with the areas used to determine cell number highlighted.

2.1.5 Mycoplasma testing

Cell lines were routinely examined for mycoplasma infection using the MycoAlert Mycoplasma Detection Kit (LONZA, Berkshire, England). This involved testing one week after retrieval from frozen storage and subsequently every three weeks. Cell culture supernatant (1.5ml) was transferred from tissue culture flasks to a microcentrifuge tube and centrifuged at 1,500rpm for 5 minutes. Following removal of cells and debris, 75µl of cleared supernatant was transferred into a white walled 96 well plate. An equal quantity of reconstituted MycoAlert Reagent was added to well and allowed to incubate for 5 minutes. The plate was read using Victor Luminometer (BMG Labtech, Brennan & Company, Dublin, Ireland). Immediately after the plate was read, 75µl of MycoAlert Substrate was added and incubated for 10 minutes. The plate was read for a second time. The ratio of the second reading to the first reading was then calculated. A ratio of greater than 1 indicates contamination in the sample.

2.2 Bile acid treatment

Cell lines were exposed to bile acids solubilised in DMSO or where the sodium salt version of the bile acid was used, in the relevant experimental media. Exposure to bile salts was carried out in experimental media without supplements, FCS or antibiotics. Matched time point controls treated with DMSO only were included as controls.

2.3 Gene expression microarray analysis

A microarray study had previously been carried out in the laboratory to identify DCA responsive genes in two oesophageal cell lines representing either end of the oesophageal carcinogenic sequence: Het-1A (normal squamous) and SKGT4 (adenocarcinoma). Microarray datasets established from patient samples were obtained from the online repository, Gene Expression Omnibus (GEO) (Kimchi *et al.*, 2005) and directly from authors who had previously published microarray studies (Ostrowski *et al.*, 2007). Microarray analysis was carried out using GeneSpring™ 7.0 software. The data were normalised and filtered (Figure 2.2). Transcripts marked as absent were removed, as were transcripts with a less than 1.5 fold difference in expression levels. Statistical significance was determined using analysis of variance (ANOVA). Resulting gene lists were comprised of genes with a high probability of changes in gene expression. A meta-analysis was carried out which combined published microarray data from patient samples with the data generated in our microarray experiments. Genelists were combined using Venn diagrams and resulting subsets were stored. This meta-analysis created a number of subsets of genelists. Network and pathway analysis was carried out on these gene lists using Ingenuity Pathways Analysis™ (IPA) (Ingenuity Systems Inc., Redwood City, CA, USA) software and MetaCore™ (Genego, Michigan, USA).

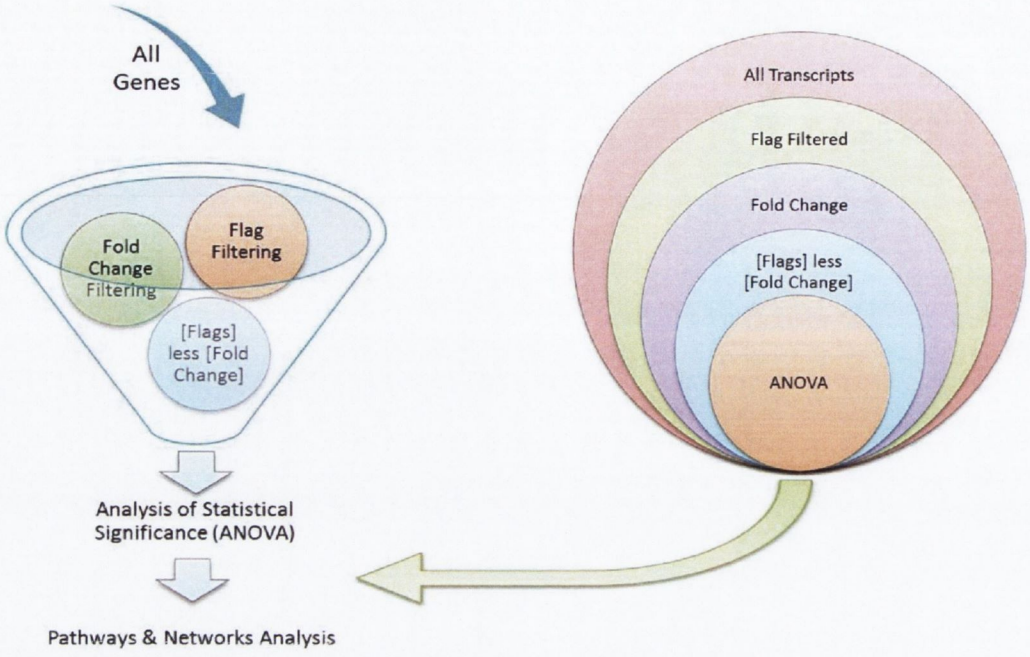


Figure 2.2: Flow through of microarray analysis

2.4 Cell viability assay

Cell viability was assessed using an MTT cell viability assay (ATCC, Middlesex, UK). The MTT ((3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) tetrazolium salt is reduced from a yellow to a purple colour by metabolically active cells. MTT reagent (10µl) was added to cells (in 100µl media). Following 2.5 hours incubation with the MTT reagent (in the incubator), 100µl of detergent reagent was added in order to lyse the cells. Cells are allowed to lyse in the dark for 2 hours at 37°C or overnight at room temperature and absorbance is read at 570nm (plate reader: Versa Max, Molecular Diagnostics, Kent, UK).

2.5 Chemical inhibitor treatment

Cells were pre-treated with each chemical inhibitor for 30 minutes followed by co-treatment with the chemical inhibitor and the bile acid over a series of time points in serum free media. Chemical inhibitors and the ranges of concentrations that these inhibitors were used are described in Table 2.2.

Table 2.2: Panel of Chemical Inhibitors used and range of concentrations

Inhibitor Target	Inhibitor	Concentration Range (μM)
BCL-XL	BH3I-1	0-100
EGFR/ErbB2	EGFR/ErbB2 Inhibitor	0-100
BCL-2	HA14-1	0-100
Jak Family	AG490	0-100
PI3K	Wortmannin	0-10
NF-κB	Bay 11-7085	0-100
PKC	Bisindolylmaleimide I	0-50
MEK	PD98059	0-100
MEK	U0126	0-10

2.6 Transfection of cells with siRNA

Small interfering RNAs (siRNAs) (siGENOME-SMARTpool; Medical Supply Company, Dublin, Ireland) were transfected into cell lines using DharmaFECT (Medical Supply Company, Dublin, Ireland) siRNA transfection reagent. Transfection reagent and siRNA were prepared in separate tubes in serum and antibiotic free media and left for 5 minutes. Transfection complexes were formed by addition of the siRNA mixture to the transfection reagent and incubated at room temperature for 20 minutes. Transfections were carried out in 96 well plates. A reverse transfection method was employed where 20μl of transfection complex was placed into the well followed by 80μl of cells. Media was changed after 48 hours and replaced with complete media for a further 24 hours, giving a total transfection time of 72 hours.

The siRNA transfection conditions were optimised using control siRNAs. The control siRNA used were a non-targeting negative control siRNA (siNT) and a positive control (siTOX) that reduces cell viability. The factors that were optimised were type of transfection reagent used (DharmaFECT 1, 2, 3 or 4; Medical Supply Company, Dublin, Ireland); concentration of transfection reagent and concentration of siRNA per well. Off-

targets effects were determined using siNT and efficiency was assessed using siTOX. The siRNA transfection conditions were determined for each cell line Table 2.3.

Table 2.3: Optimised siRNA transfection conditions for oesophageal cell lines

Cell Line	Transfection Reagent (TR)	Quantity of TR per well	Concentration of siRNA per well
GOhTRT	DharmaFECT 4	0.4µl	100nM
Het-1A	DharmaFECT 1	0.4µl	100nM
OE33	DharmaFECT 3	0.15µl	100nM

2.7 RNA extraction

RNA extraction was carried out using the RNeasy Plus Mini Kit (Qiagen, West Sussex, UK) as per manufacturer's instructions. Briefly, genomic DNA (gDNA) was removed from the sample by passing the cell lysate through a gDNA eliminator column which bound the gDNA. RNA was precipitated from the remaining flow through using 70% ethanol. This RNA was then bound to a purification column. Following a series of washing steps, purified RNA was eluted using RNase-free water. RNA was quantified using a NanoDrop Spectrophotometer (NanoDrop, Technologies, Wilmington, DE).

2.8 Real time reverse transcription-polymerase chain reaction

RNA was reverse transcribed using the RETROscript RT-PCR kit (Ambion/Applied Biosystems, Warrington, UK). Two micrograms of RNA was heat denatured at 70°C for 3 minutes followed by annealing with the oligo deoxythymidine primer by cooling to 42°C. Real time PCR was carried out in 384-well clear reaction plates using TaqMan® Gene Expression Assay (Applied Biosystems, Foster City, CA) with the ABI Prism 7900T real time thermocycler (Applied Biosystems, Foster City, CA). TaqMan® Gene Expression Assays contain an unlabelled PCR primer pair and a dye labelled probe containing a

nonfluorescent quencher. The labelled probe binds to target DNA between the two unlabelled PCR primers. During PCR, Taq polymerase, directed by the template strand, extends the unlabelled primers. The labelled probe is cleaved by the polymerase which releases the quencher allowing the dye to fluoresce. The level of fluorescence detected reflects the expression level of the target DNA.

Fold change in gene expression was calculated using the $^{\Delta\Delta}$Ct relative expression method where glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an endogenous control and untreated resting samples were used as the calibrator for fold change. GAPDH was used as the endogenous control in this body of work as previous work in this laboratory demonstrated a consistent level of GAPDH gene expression during the cell cycle of each cell line under investigation.

The relative quantification analysis technique was used in these experiments to determine expression levels of the genes of interest. This method involved a comparison of the cycle threshold (Ct) value of the gene of interest with the Ct value of an endogenous control gene, in this case GAPDH to obtain a delta Ct value (ΔCt). The fold change of the gene of interest was derived from the delta-delta Ct value ($\Delta\Delta$Ct). The $\Delta\Delta$Ct value represents the change in ΔCt of the gene of interest between a sample that has been exposed to a treatment and a resting untreated sample which is used as a calibrator (Livak and Schmittgen, 2001). The real time PCR protocol that was implemented in this case involved 40 cycles. The Ct value obtained for each gene in the sample is resultant from the initial copy number of that gene in the original sample. The lower the Ct number of the gene of interest, the higher the expression level of the gene.

2.9 siRNA Library Screen

2.9.1 Assessment of consistency & accuracy of liquid handling robots

Fluorescein standard curves were acquired using a Gilson RepeatMan (Anachem, Luton, UK) positive displacement pipette, a gold standard in consistency and accuracy. A number of fluorescein concentrations were assessed with 0.1µg/ml being chosen as this concentration gave a straight line when quantity dispensed was plotted against relative fluorescent units (RFUs). RFUs were determined using a FLUOstar OPTIMA (BMG Labtech, Brennan & Company, Dublin, Ireland). Fluorescein has an absorption maximum at 494nm and an emission maximum of 521nm.

The consistency and accuracy of each of the 96 needles of the Matrix Hydra II liquid handling robot (Thermo Scientific, Barrington, IL, USA) were assessed by dispensing 3µl of fluorescein a total of 10 times. These values were plotted on a surface graph using Microsoft Excel™ 2010 to visualise each needle to ensure consistency across the plate. The average volume dispensed by each needle was determined. The percentage coefficient of variance for each needle was determined using the following formula: $\left(\frac{SD}{Mean}\right) \times 100$; where SD is the standard deviation of the sample. These values were also plotted on a surface graph using Microsoft Excel™ 2010 to visualise similar levels of %CV across the 96 needles. Standard curves were determined for the Matrix™ Hydra II (Thermo Scientific, Barrington, IL, USA) and the Matrix WellMate (Thermo Scientific, Barrington, IL, USA) and compared to the standard curve of the Gilson RepeatMan (Anachem, Anachem, Luton, UK) pipette using Pearson's correlation coefficient determined using GraphPad™ Prism 5.

The accuracy of the WellMate™ was assessed using a gravimetric method. This involved dispensing 50µl of water from each tube of the dispensing mechanism into a single microcentrifuge tube ten times to give an expected total of 500µl in the microcentrifuge.

This was repeated three times for each tube. The mass of the water dispensed was used to estimate the volume dispensed using the formula: $D = \frac{m}{V}$; where D = Density; m = mass, v = volume (Density of water was taken as 1g/ml).

2.9.2 Automation of siRNA transfection protocol

Each step of the siRNA transfection protocol was upscaled and automated using the Matrix Hydra II™, the Matrix WellMate™ or the Gilson RepeatMan™. The automated steps of the protocol are illustrated in Figure 2.3.

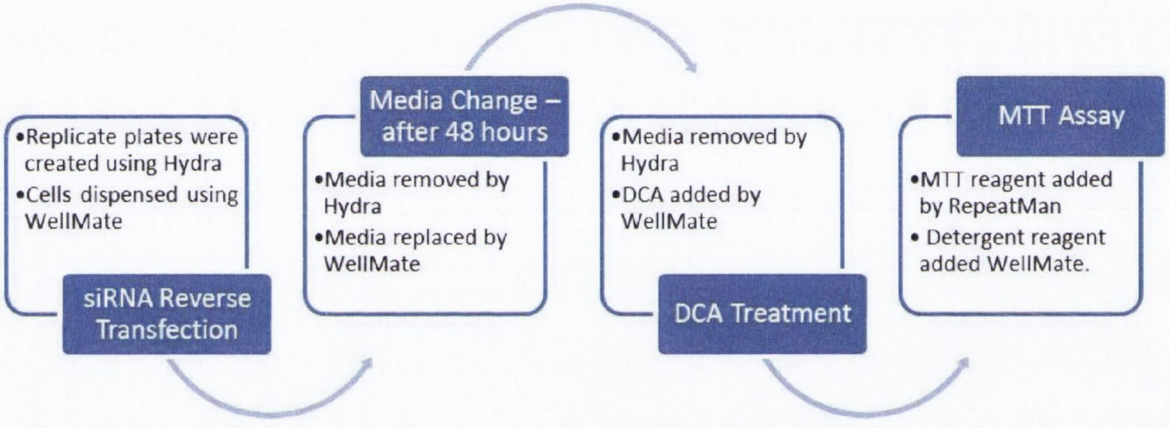


Figure 2.3: Automated siRNA transfection protocol

2.9.3 Plate layout

The siRNA library plates were available in a 96 well format from Dharmacon (Medical Supply Company, Dublin, Ireland). Each plate had 16 empty wells to accommodate screen specific controls. The plate layout implemented in this screen is illustrated in Table 2.4. Positive and negative controls are included on each row to allow for potential row to row variation. The controls were also staggered to eliminate potential influence of possible effects.

Table 2.4: siRNA library screen plate including screen specific controls

N1	P1	S1	S9	S17	S25	S33	S41	S49	S57	S65	S73
P2	N2	S2	S10	S18	S26	S34	S42	S50	S58	S66	S74
N3	P3	S3	S11	S19	S27	S35	S43	S51	S59	S67	S75
P4	N4	S4	S12	S20	S28	S36	S44	S52	S60	S68	S76
N5	P5	S5	S13	S21	S29	S37	S45	S53	S61	S69	S77
P6	N6	S6	S14	S22	S30	S38	S46	S54	S62	S70	S78
N7	P7	S7	S15	S23	S31	S39	S47	S55	S63	S71	S79
P8	N8	S8	S16	S24	S32	S40	S48	S56	S64	S72	S80

N = neg control; P = pos control; S = siRNA

2.9.4 Quality assurance/quality control

The quality of the data generated from the siRNA library screen targeting GPCRs was determined using three independent methods. The first approach was to identify outliers using Grubb's Test for Outliers which is freely available at www.graphpad.com/quickcalcs/Grubbs1.cfm. Boxplots was created for each replicate of each plate to facilitate a visual illustration of the descriptive statistics of each replicate of plate. The range of the distribution of the values and the median value on each plate were represented in the boxplots. Scatterplots were created to demonstrate correlation between each of the replicates.

2.9.4.1 Identification of outliers

During the course of the experiment, a number of potential outliers were identified. These included wells which exhibited an intense purple colour following MTT incorporation and cell lysis. This may be the result of an infection in the well in question. As described above, Grubb's test for outliers was used.

Grubb's outlier test determines if all values in the sample are likely to come from the same Gaussian distribution population. This test quantifies how far an individual value is from the other values in the sample. A value known as Z was determined, which is the ratio

between the value in question and the sample mean divided by the sample standard deviation. A large Z value indicates that the data point in question is located far from the rest of the values in the sample. Tables of critical values for Z have been created which are based on sample size. If a Z value that is derived for a sample data point is greater than the critical Z value determined for a sample of similar size, it is unlikely to be from the same Gaussian distribution as the rest of sample. From this point onwards all calculations and graphical representations of data are with the outliers removed.

2.9.5 siRNA screen analysis strategy

The aim of the siRNA library screen was to sensitise GOhTRTs to DCA induced cell death following specific inhibition of a target using siRNA technology. The library screen that was used targeted GPCRs. Two analysis strategies were implemented to identify the top targets: cell viability and z-score.

2.9.5.1 Cell viability

The cell viability of each well was assessed using an MTT cell proliferation assay. Cell viability was expressed as a percentage of a non-targeting negative control siRNA (siNT) was determined on a plate by plate basis using the following formula: $\frac{abs\ of\ target\ well}{median\ abs\ of\ neg\ control\ well} \times 100$. Each replicate well representing one siRNA target was divided by the median of the negative controls from all replicates of that plate and expressed as a percentage. The median was chosen to minimise possible influence of outliers from within the sample of negative controls. The Student's t-test was used to determine significance.

2.9.5.2 z-score

The z-score is the distance a value lies from the mean of the sample measured in units of standard deviation. The z-score of each value was determined using the following formula: $z = \frac{x-\mu}{\sigma}$; where x = value; μ = sample mean; σ = standard deviation.

2.10 Cocktail of bile salts

In order to recreate physiological conditions more accurately *in vitro*, cell lines were exposed to a cocktail of bile salts which had been derived from clinical studies examining refluxate components in patients suffering from GORD. The cocktail was comprised of the following conjugated bile acids: glycocholic acid, taurocholic acid, glycochenodeoxycholic acid, taurochenodeoxycholic acid, glycodeoxycholic acid and taurodeoxycholic acid in a 20:3:15:3:6:1 ratio of molar concentration (Huo *et al.*, 2010, Nehra *et al.*, 1999, Kauer *et al.*, 1997). A fresh cocktail was prepared each day.

2.11 Acidification of experimental media

Media was acidified using concentrated HCl. HCl was added to the media while the pH was constantly being read using a pH meter. Acidified media was made up fresh each day.

2.12 Pulsatile treatment of oesophageal cells

In order to further mimic physiological conditions *in vitro*, oesophageal cell lines were exposed to bile salts and low pH media in ten minute pulses, three times a day for three or seven days. The daily pulses were given over a 12 hour period. Generally, the first pulse was administered at 08.00hrs, the experimental media was replaced with complete media. The second and third pulses were administered at 14.00hrs and 20.00hrs respectively. The

cells were allowed to recover in complete media following each pulse. RNA was collected following the final pulse unless otherwise stated.

2.13 Detection of protein levels in oesophageal cells

2.13.1 Protein extraction

Protein was extracted from cells using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Medical Supply Company, Dublin, Ireland) as per manufacturer's instructions. Briefly, cells were harvesting in HBSS following mechanical disruption of the adherent cells. Cells were pelleted in a 1.5ml microcentrifuge tube for 5 minutes at 1,500 rpm. Ice-cold CER I reagent was added to the cell pellet, the tube was vortexed (15 seconds) and incubated on ice for 10 minutes. Ice-cold CER II was added, vortexed (5 seconds) and incubate on ice for 1 minute. The sample was then centrifuged for 5 minutes at maximum power. The supernatant, which contains the cytoplasmic fraction, was transferred to a pre-chilled tube and stored at -20°C. The remaining pellet was suspended in ice-cold NER reagent, vortexed (15 seconds) and incubated for 10 minutes. The tube was vortexed (15 seconds) every ten minutes for a total of 40 minutes. The tube was centrifuged at maximum power for ten minutes. The supernatant, containing the nuclear fraction was stored in a pre-chilled tube at -20°C.

2.13.2 Protein quantification

Protein was quantified using a Micro BCA Protein Assay Kit (Pierce/Medical Supply Company, Dublin, Ireland) as per manufacturer's instructions. Briefly, 10µl of the extracted protein fractions was diluted to 450µl with ultra-pure water. A standard curve using known concentrations of bovine serum albumin (BSA) was prepared in ultra-pure water. The extraction reagents, CER I and NER were also quantified to facilitate the removal of background values resulting from the extraction reagents - 10µl of each reagent were made diluted to 450µl with ultra-pure water. Micro BCA working reagent (WR) was

prepared using the following formula to calculate the final quantity required: (# standards + # unknowns) x (# replicates) x volume WR per sample. Each sample was tested in triplicate using 150µl of each sample with 150µl WR per replicate. The WR was comprised of reagent MA, reagent MB and reagent MC in a ratio of 25:24:1 respectively. The samples were incubated with WR in the dark for 2 hours at 37°C. The absorbance at 562nm was measure using a Versa Max plate reader (Molecular Devices, Kent, UK). The concentration of unknown samples was estimated using the BSA standard curve.

2.13.3 Acetone precipitation

The protein was precipitation and concentrated from the extraction buffer using acetone in a 5:1 ratio acetone:sample. Ice-cold acetone was added to the protein fraction and incubated at -20°C for 30-40 minutes with gentle agitation approximately every 5 minutes. Following concentration of the protein using acetone, the tube was centrifuged at 6,500rpm for 5 minutes. The supernatant was removed and the pellet of protein was resuspended in 40µl sample buffer (Sigma, Poole, Dorset, UK). The protein was heated at 100°C for 5 minutes.

2.13.4 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Molecular weight markers (Sigma, Poole, Dorset, UK) were vortexed and heated to 100°C for 5 minutes. Resolving and stacking gels were prepared as per Table 2.5. Each lane of stacking gel was loaded with 40µl of protein extract in sample buffer or molecular weight marker. Sample buffer was added to lanes that did not contain protein to ensure an even running of the samples. The gels were run at 30mA per gels in 1X running buffer (10X Running Buffer: 30g Tris Base; 144g Glycine; 10g SDS – made up to 1 litre with ultra-pure water). The gel was allowed to run until the sample buffer has reached the bottom of the gel.

Table 2.5: Resolving and Stacking Gels utilised for SDS-PAGE were prepared as described below

	Stacking Gel	Resolving Gel
Acrylamide/Bisacrylamide	1.33ml	6.66ml
1.0M Tris (pH 6.8)	3.05ml	-
1.5M Tris (pH 8.8)	-	5.0ml
Distilled Water	5.55ml	8.23
APS (10%)	50µl	100µl
TEMED	10µl	10µl

The proteins on the gel were transferred to PVDF membrane using a semi-dry transfer method. The lane containing the molecular weight marker was removed from the membrane and stained with Commaasie Blue, followed by destaining. The gels were allowed to transfer for 1 hour at 100mA per gel in 1X transfer buffer (1X Transfer Buffer: 5.8g TRIS, 2.9g Glycine, 4mls 10% SDS & 200mls Methanol; made up to 1 litre with ultra-pure water).

The membrane was incubated in 5% Marvel in PBS with gentle shaking for 30-40 minutes to block non-specific binding sites. The primary antibody was prepared in 5% marvel in PBS. The concentration of each antibody was optimised for each cell line. The membrane was incubated with the primary antibody at 4°C overnight with gentle shaking. Following incubation with the primary antibody, the membrane was washed 3 times for 5 minutes each wash in PBS tween. The membrane was then incubated with the relevant horseradish peroxidase (HRP) conjugated secondary antibody for 1 hour with gentle shaking at room temperature. Blots were washed three times for five minutes each wash and stored in PBS at 4°C. Bands were visualised using a ImageQuant LAS 4000 chemiluminescent imaging system (GE Life Sciences, Brennan & Company, Dublin, Ireland).

Chapter 3

Results

The Identification of Deoxycholic Acid Responsive Genes Altered in Oesophageal Carcinogenesis using Gene Expression Microarray Analysis

3 Gene Expression Microarray Analysis

3.1 Introduction

The oesophageal carcinogenic sequence is an established multistage process that involves sequential progression through a number of defined stages (Jankowski *et al.*, 1999). Each stage of oesophageal carcinogenesis is well defined phenotypically but a clear insight into the underlying molecular mechanisms involved in disease progression has not been well characterised. Gastro oesophageal reflux disease (GORD) is considered to be the highest risk factor associated with the development of Barrett's oesophagus (Lagergren *et al.*, 1999). Bile acids present in refluxate are associated with oesophageal mucosal injury and subsequent development of intestinal-like metaplasia followed by progression to cancer (Guillem, 2005). A number of cancer related phenotypes are induced by bile acids. Bile acid exposure has been demonstrated to result in DNA damage (Zhang et al., 2009a) and a potential role in inflammation has also been suggested (Jenkins *et al.*, 2002). As inflammation has recently been proposed as the seventh hallmark of cancer, a potential role for bile acids to act as carcinogens in the oesophagus is becoming more likely through the induction and promotion of inflammation (Colotta *et al.*, 2009). This study hypothesises that exposure of the oesophagus to bile acid induces a milieu of transcriptional responses that may contribute to the development of oesophageal adenocarcinoma (OAC) and its preliminary lesions at a molecular level. It is also thought that genetic anomalies accrued in preliminary lesions may alter molecular responses to bile acid exposure thus subverting them in the propagation of carcinogenesis. This main objective of this study was to identify genes involved in oesophageal carcinogenesis that are also regulated by bile acids.

Microarray gene expression technology was utilised to explore and develop this hypothesis. Gene expression analysis using microarray technology is a powerful tool to study genotypic changes of cells in response to external stimuli or differences in tissues

representing various disease stages. Advances in microarray gene expression technology have laid the foundation for unprecedented advances in investigations of the underlying molecular mechanism of various diseases (Altshuler *et al.*, 2008). In the case of oesophageal pathogenesis, gene expression profiling using microarray technology has previously demonstrated that Barrett's oesophagus (BO) represents an intermediate stage in oesophageal cancer development at a molecular level (Wang *et al.*, 2006). Data from previously published microarray studies are publically available in databases such as the Gene Expression Omnibus (GEO). The GEO repository is a database of high density gene expression data from published microarray studies. Microarray gene expression studies often generate more data than is relevant to the original hypothesis under investigation. The GEO repository archives high density gene expression data, allowing raw data to be accessed and downloaded for inclusion in future studies.

The bile acid deoxycholic acid (DCA) has been associated with oesophageal carcinogenesis in a number of studies. Patients with reflux containing both gastric and duodenal components have been associated with more severe mucosal injury than counterparts with gastric juice component only (Kauer *et al.*, 1997). A significantly higher total bile acid concentration has been determined in oesophagitis and BO patients compared to patients with minimal injury (Nehra *et al.*, 1999). Development of oesophageal adenocarcinoma requires the accumulation of genetic mutations that confer a competitive advantage to cancer cells. Bile acids have been implicated in oesophageal adenocarcinoma development and further investigation into the transcriptional response of oesophageal cell lines to bile acid exposure would yield a greater insight into the underlying molecular mechanisms of oesophageal adenocarcinoma development. Increased understanding of these mechanisms may lead to the development of potentially novel therapeutic and prevention strategies. DCA has previously been demonstrated to

display many carcinogenic properties including the ability to induce DNA damage (Clemons *et al.*, 2007) and inflammation (Oyama *et al.*, 2005).

A comparative bioinformatics strategy was developed combining data generated by an original microarray study examining response of cell lines *in vitro* to DCA with previously published datasets charting oesophageal disease progression in patient samples. A cohort of genes was identified following the reanalysis of two published patient studies which contained genes with altered expression profiles during oesophageal cancer development (Kimchi et al., 2005, Ostrowski et al., 2007). Both of these cohorts were then compared to a set of DCA responsive genes identified in this original microarray study. Genes displaying differential expression patterns throughout the oesophageal carcinogenic sequence that additionally demonstrated cell type specific responses to bile acid exposure were identified. The cohort of genes would reveal additional insight into the underlying mechanisms of DCA signalling in oesophageal carcinogenesis. This comparative informatics analysis successfully integrated previously published patient microarray data with an original gene expression study. This approach enhanced the impact of both the published microarray studies and the original microarray experiment that was carried out.

Genes of interest that were identified were then analysed using a systems biology approach. Cellular pathways and processes associated with DCA responsive genes with altered gene expression profiles in progression through the oesophageal carcinogenic sequence were identified. This analysis method identified direct mechanisms by which bile acid signalling promotes the development of oesophageal adenocarcinoma. The integration of these microarray studies demonstrate that bile acids have an integral role to play in oesophageal carcinogenesis.

3.2 Objectives

The main objective of this study was to determine altered gene expression profiles during oesophageal carcinogenesis and to investigate the association of DCA regulated genes with this process. This aim was achieved through:

- the identification of DCA transcriptional responses in Het-1A and SKGT4 oesophageal cell lines.
- the determination of genes with altered expression profiles during oesophageal carcinogenesis through reanalysis of published oesophageal patient microarray datasets.
- the development of a comparative bioinformatics strategy to compare and contrast these gene cohorts.
- highlighting the loss or gain in expression of DCA regulated genes during oesophageal cancer development.
- the preliminary investigation of potential pathways and processes through which bile acids may contribute to progression through the oesophageal carcinogenic sequence using a systems biology approach.

3.3 Results

3.3.1 Gene expression microarray analysis strategy

This study developed a gene expression microarray analysis strategy to identify a cohort of DCA responsive genes in oesophageal cell lines. This study also aimed to identify a dataset of genes with altered expression profiles throughout the oesophageal carcinogenic sequence utilising previously published gene expression studies. The original data from this study of the transcriptional response of oesophageal cell lines following DCA exposure is available from the GEO database, accession number GSE13400 (Figure 3.1A & C; www.ncbi.nlm.nih.gov/geo/). The GEO database was further utilised to access a

previously published microarray studies analysing gene expression profiles during progression through the oesophageal adenocarcinoma sequence. The raw data from this study was available from the GEO database (GDS1321) (Kimchi *et al.*, 2005) (Figure 3.1B).

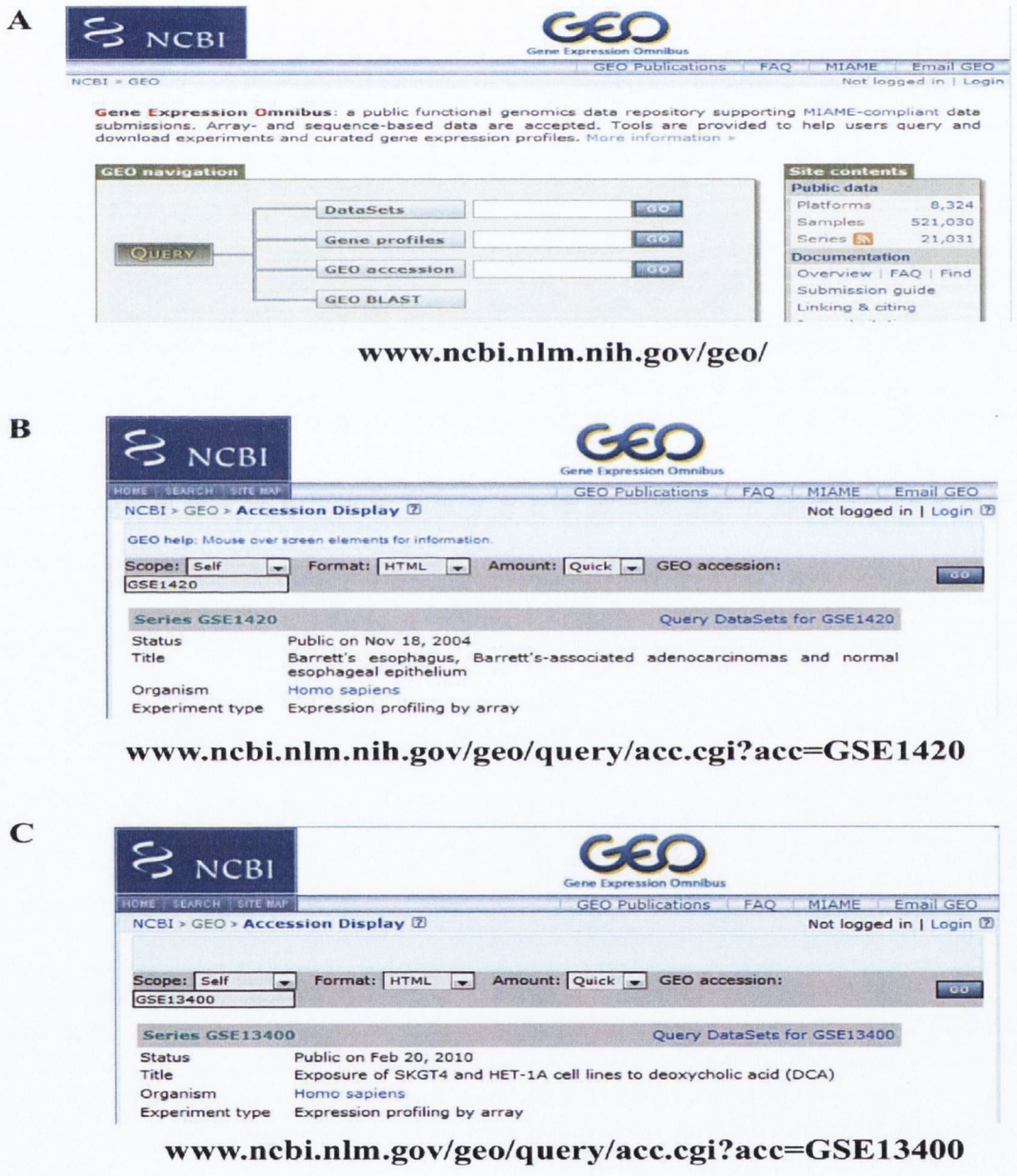


Figure 3.1: Gene Expression Omnibus (GEO) Website. (A) The gene expression omnibus (GEO) database is a repository for high throughput gene expression data. Two of the datasets used in this study are freely available in the GEO database (www.ncbi.nlm.nih.gov/geo/). (B) The Kimchi *et al* (2005) data is available under the dataset identification number GDS1321 (Kimchi *et al.*, 2005). (C) The DCA responsive genes in Het-1A and SKGT4 cells was published by Duggan *et al* with the series identification number GSE13400 (Duggan et al., 2010).

The cell lines utilised in this study represented normal oesophageal squamous cells (Het-1A) and the final stage of the oesophageal carcinogenic sequence, adenocarcinoma (SKGT4). The use of both these cell lines aimed to provide a comprehensive overview of the difference in transcriptional responses to DCA exposure in cell lines representing either end of the oesophageal carcinogenic sequence. Two previously published microarray studies charting oesophageal carcinogenesis were included in this study (Kimchi et al., 2005, Ostrowski et al., 2007). Genes with altered expression profiles throughout the oesophageal carcinogenesis were identified in both oesophageal patient studies following a reanalysis of the raw data.

An analysis strategy was developed to identify transcriptional alterations in each of the three data sets (Figure 3.2). This strategy involved filtering and statistical analysis of the raw data to acquire a set of transcripts with significantly altered gene expression profiles in response to DCA exposure or resulting from progression through the carcinogenic sequence.

Each microarray study was carried out using different Affymetrix microarray formats which accounts for the varied number of initial transcripts between each of the studies. This study investigating DCA exposure of oesophageal cell lines used a U133A plus 2.0 format (54 675 transcripts) (Duggan et al., 2010), the Kimchi *et al* (2005) study used the U133A format (22,283 transcripts) and the Ostrowski *et al* (2007) study used the U133A 2.0 format (22,277 transcripts) (Kimchi *et al.*, 2005, Ostrowski *et al.*, 2007). The later versions of this microarray format contain the same probe set as the earlier versions with the addition of transcripts to detect a larger of genes (Affymetrix, 2003). The first steps of this strategy involved filtering based on flags and fold changes. Flags represent the quality of the signal from each of the transcripts. A transcript with a low signal will be marked as

'absent'. The transcripts that were flagged 'absent' at all-time points or all stages of disease were removed (Het-1A - DCA responsive genes: 27 910; SKGT4 - DCA responsive genes: 24,339; Kimchi *et al* (2005) study - 17,132; Ostrowski *et al* (2007) study - not available). A fold change of greater than 1.5 between control and treated samples or between normal and disease samples was deemed significant for this study (Het-1A - DCA responsive genes: 15,995; SKGT4 - DCA responsive genes: 15,093; Kimchi *et al* (2005) study - 8,235; Ostrowski *et al* (2007) study - 18,435). Transcripts with fold changes less than 1.5 were removed from the flag filtered subset (Het-1A - DCA responsive genes: 11,915; SKGT4 - DCA responsive genes: 9,246; Kimchi *et al* (2005) study - 8,897; Ostrowski *et al* (2007) study - 3,842). The remaining genes were analysed for statistical significance using t-tests of analysis of variance (ANOVA; p-value < 0.05) (Het-1A - DCA responsive genes: 7,667; SKGT4 - DCA responsive genes: 4,673; Kimchi *et al* (2005) study - 3,011; Ostrowski *et al* (2007) study - 3,817) (Figure 3.3).

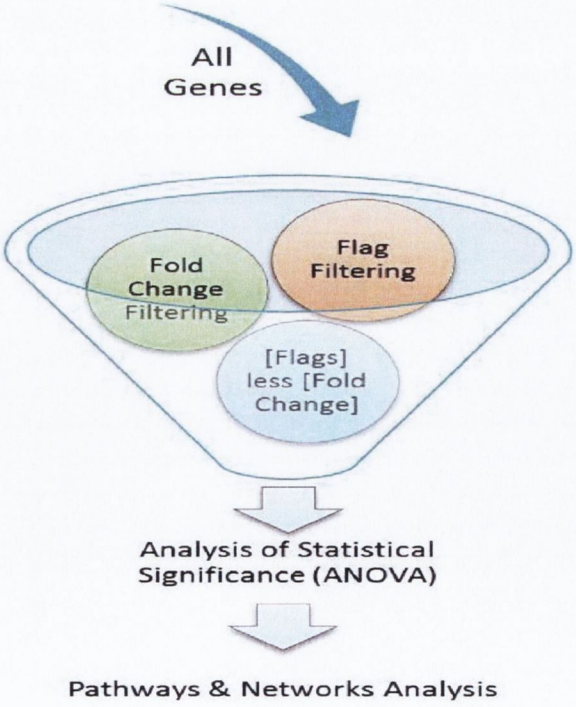
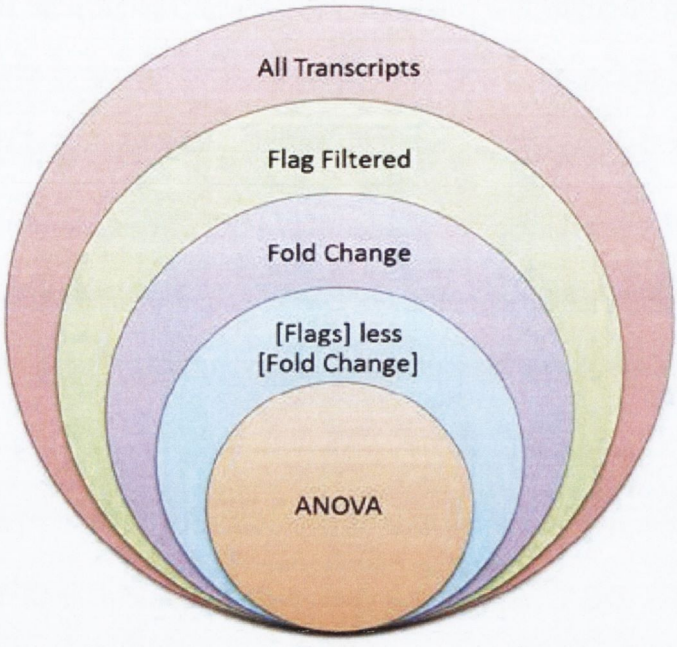


Figure 3.2: Gene Funnelling of Microarray Data. Microarray data was filtered based on fold change and flags (present or absent). Transcripts flagged as present and with a fold change greater than 1.5 were analysed for statistical significance using t-tests of analysis of variance (ANOVA). Genes with statistically significant alterations of expression profiles were analysed using IPA. This software package identified cellular pathways, processes and signalling networks that were linked with the generated gene datasets.

A



B

Cell Line Data Sets		Patient Data Sets	
Het-1A - DCA Responsive	SKGT4 - DCA Responsive	Kimchi *et al* M-D-A Sequence	Ostrowski *et al* BO
54,675	54,675	22,283	22,277
27,910	24,339	17,132	22,277
15,995	15,093	8,235	18,435
11,915	9,246	8,897	3,842
7,667	4,673	3,011	3,817

Figure 3.3: Genes with statistically significant alterations in expression profiles were identified using a gene funnelling analysis strategy. (A) The first step in the analysis of the microarray data involved the removal of transcripts with signals flagged absent. Subsequent steps removed transcripts with a fold change less than 1.5. Genes with statistically significant alterations in gene expression profiles were identified using t-tests of analysis of variance (ANOVA; p-value < 0.05). (B) The analysis strategy identified 7,667 genes in Het-1A cells that were responsive to DCA. There were 4,673 DCA responsive genes identified in the SKGT4 cells with 3,011 and 3,817 altered expression profiles identified in the Kimchi *et al* (2005) and the Ostrowski *et al* (2007) studies respectively which chart progression through the carcinogenic sequence (Kimchi et al., 2005, Ostrowski et al., 2007).

3.3.2 DCA responsive genes identified in Het-1A and SKGT4 cell lines

This study aimed to investigate and compare the effect of DCA exposure on the transcriptional response of two oesophageal cell lines. The cell lines used represented normal squamous cells (Het-1A) and oesophageal adenocarcinoma cells (SKGT4). The Het-1A and SKGT4 cell lines were utilised as these cell lines represented both extremes of the oesophageal carcinogenic sequence. The transcriptional responses of these cell lines provided an overview of the changes in response to DCA exposure following transformation from normal to cancer. Both cell lines were exposed to DCA ([300μM]) for 4, 8, 12 and 24 hours. Gene expression microarrays were carried out using an Affymetrix U133A 2.0 plus array platform (54,675 transcripts) and analysis was completed using GeneSpring 7.0 software. DCA exposure [300μM] of Het-1A cells resulted in the change in expression of a total of 7,667 genes, 5,661 were specific to the Het-1A cell line whereas 2,006 genes were significantly altered in both cell lines. A further 2,631 genes were significantly altered in response to DCA in SKGT4 cells specifically (ANOVA $P < 0.05$; Bonferroni-false discovery rate; Figure 3.4 A). The gene expression profiles of these genes are illustrated in Figure 3.4 B.

The expression profiles of both shared response genes and cell line specific genes are illustrated in the context of both cell lines. These graphs demonstrate the level of expression of each gene at each DCA exposure time point. A green colour indicates a low expression level, with red colour indicating high levels of expression. Each gene is represented by one line that illustrates the expression level of the gene in question at each time point. A straight line represents no alteration in gene expression whereas a gene with an altered transcriptional response will slope up or down between each time point, depending on whether or not the gene is induced or repressed. It is evident that there is extensive transcriptional activity in response to DCA exposure in both these cell lines.

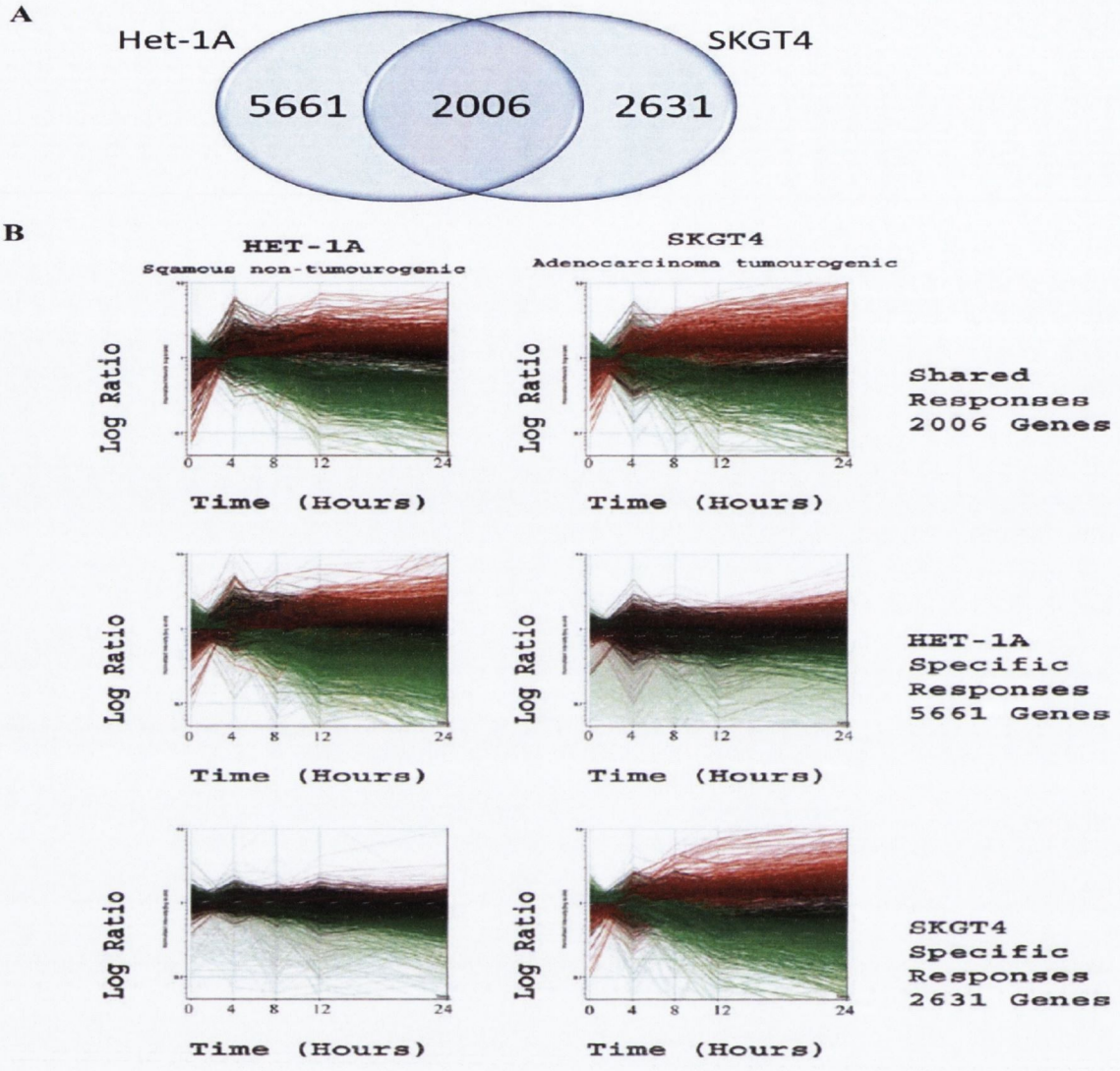


Figure 3.4: Altered gene expression profiles in response to DCA in Het-1A and SKGT4 cell lines. Het-1A and SKGT4 cells were exposed to DCA ([300μM]) over time (4, 8, 12 & 24 hrs) and altered gene expression profiles were identified. (A) A total of 2,006 genes with altered profiles were shared between the two cell lines. Cell line specific cohorts were also identified in both the Het-1A and SKGT4 cell lines with 5,661 and 2,621 gene expression profiles were altered respectively. (B) Line graphs illustrate the profile of the altered gene expression across time of DCA exposure (red = high expression; green = low expression). The further an expression level is from the baseline, the greater the difference in expression profile of the gene in response to DCA. The base line expression level is represented by a horizontal line in the centre of the graph. Shared response genes have altered genes expression profiles when displayed using values from either the Het-1A cell line (left column) or the SKGT4 cell line (right column). Genes with altered expression profiles in response to DCA in the Het-1A alone (second row, left column), do not have altered expression profiles in when displayed using expression values from the SKGT4 cell line, demonstrated by the organisation of the expression profiles close to the baseline values (second row, right column). Genes with altered expression profiles in response to DCA in the SKGT4 cell line alone (third row, right column) do not have altered expression profiles when displayed using expression values from the Het-1A cell line (third row, left column).

The dataset of genes with shared responses displayed transcriptional activity in the context of the expression values obtained in both cells lines (Figure 3.4 B). Each line representing a gene that is altered in both cell lines deviates significantly from a straight line. The Het-1A specific responses, when displayed in the context of the expression values obtained using the Het-1A cell line, exhibit varied transcriptional responses. However, when the Het-1A specific responses are displayed using the expression levels obtained in the SKGT4 cell line, each line representing the expression level of a gene does not deviate significantly from a straight line. This illustrates in a graphical form that the Het-1A specific responses are not significantly altered in the SKGT4 cell line. The converse is true with the SKGT4 specific responses (Figure 3.4).

Heat maps were generated using GeneSpring 7.0 software (Figure 3.5). Each horizontal row represents one gene across each of the treatment conditions. Each column represents one treatment, in this instance length of time of DCA exposure of each cell line. A green colour represents low expression levels with red indicating high expression levels. A detailed heat map containing genes with altered expression in Het-1A and SKGT4 cells specifically and the shared response is illustrated in Figure 3.6. It is evident from this heat map that the Het-1A specific responses do not have altered expression in SKGT4 cells following exposure to DCA. Each horizontal line representing expression of a single gene displays altered gene expression depicted by a change in colour only in Het-1A samples that have been exposed to DCA with little or no colour change in the samples from the SKGT4 cells (Figure 3.6 A). The shared response genes clearly demonstrated altered gene expression profiles in both cell lines, as the expression level of each gene changes over time of DCA exposure in both the Het-1A and SKGT4 cell lines (Figure 3.6 B). The altered gene expression profiles of SKGT4 specific responses are also evident (Figure 3.6 C).

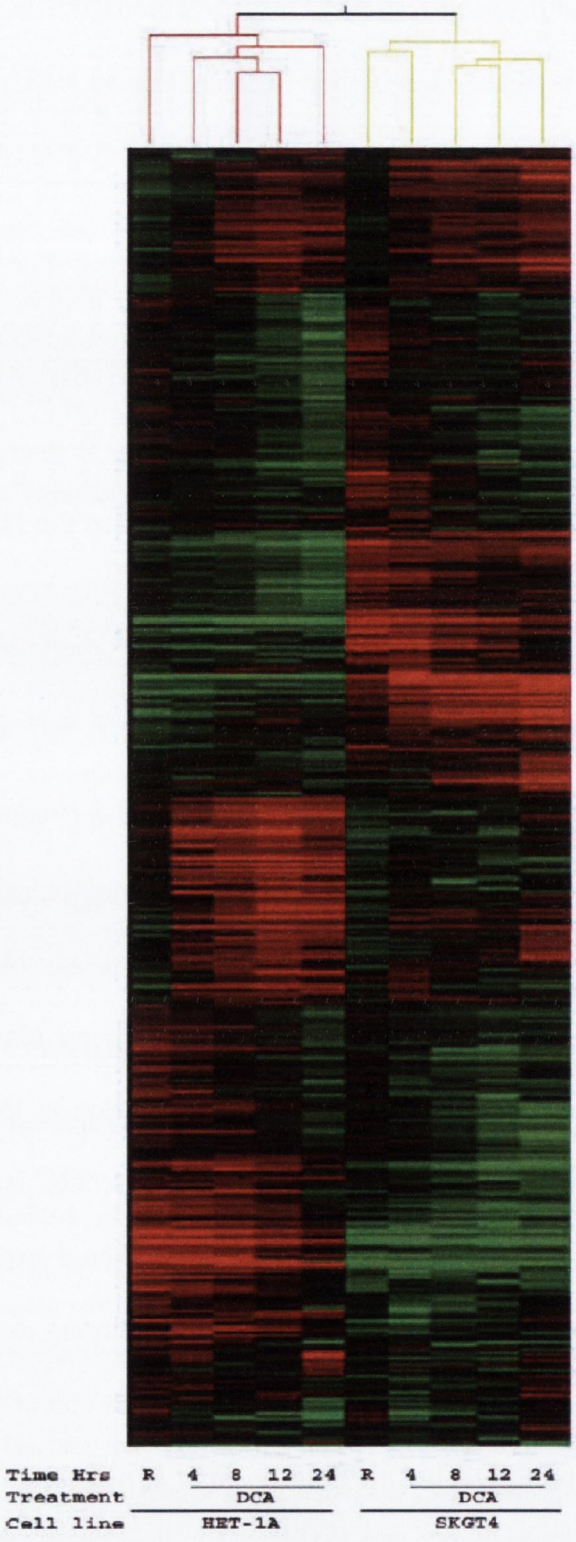


Figure 3.5: Genes with statistically significant alterations in DCA exposed Het-1A and SKGT4 cells. Gene expression profiling was carried out using a DNA microarray on Het-1A and SKGT4 cells following exposure to DCA for 4, 8, 12 and 24 hours. Significantly altered gene expression profiles were identified using the strategy described in Figure 3.2 and displayed using a traditional heat map. High expression levels are coloured red with green representing low expression.

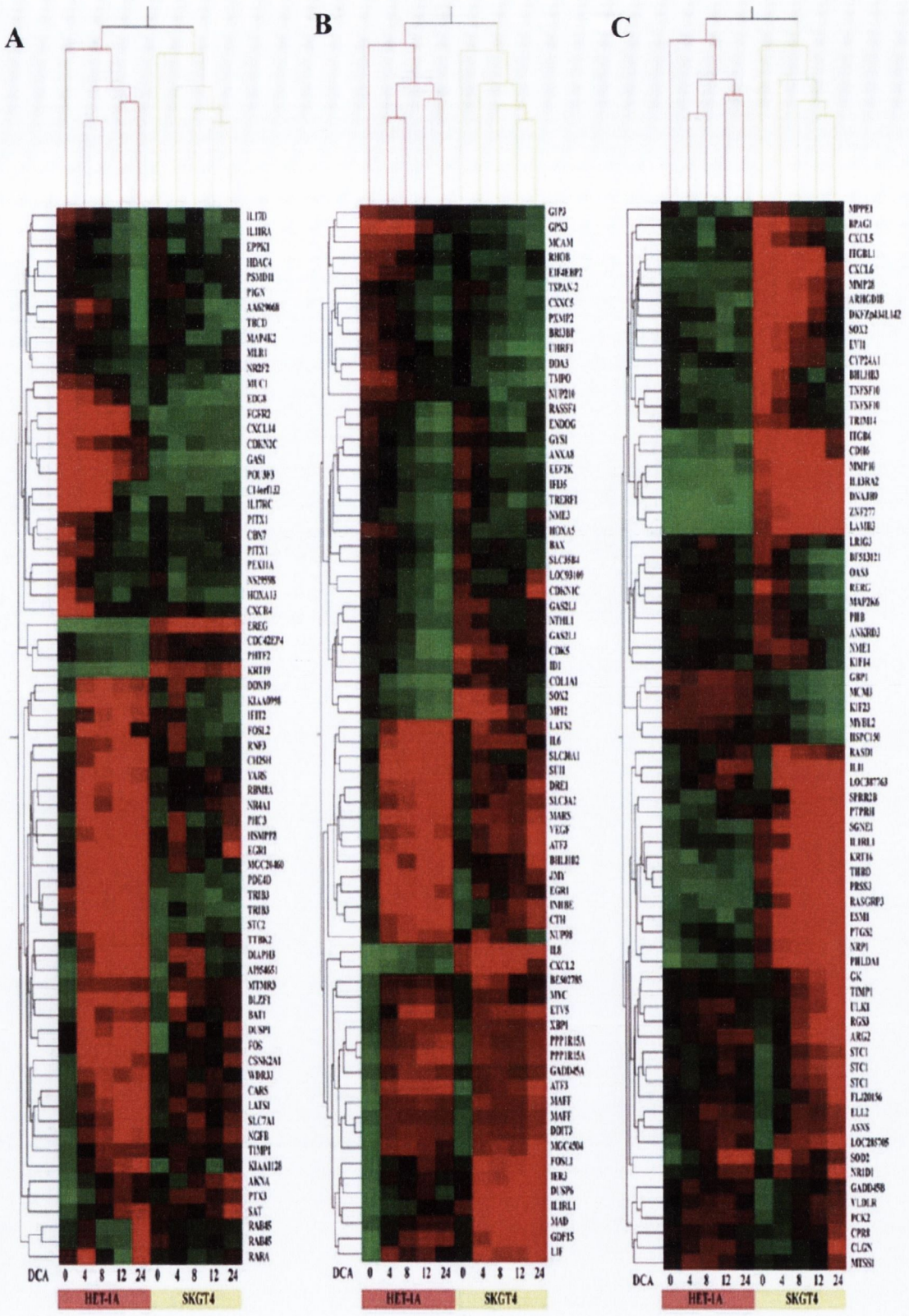


Figure 3.6: Significantly altered gene expression profiles in DCA exposed Het-1A and SKGT4 cells. Altered gene expression profiles were ranked by p value to identify the most significant (A) Het-1A specific, (B) shared and (C) SKGT4 specific responses to DCA exposure and displayed in the above heat-map. Cell line specific responses display altered gene expression profiles i.e. colour changes, only in the relevant DCA exposed cell lines.

3.3.3 Altered gene expression profiles identified during oesophageal cancer development

The GEO database is a valuable source of previously published high density expression data and currently holds 59 oesophageal related microarray studies. This study aimed to identify genes with altered gene expression profiles in patient samples charting oesophageal carcinogenesis. The datasets that were chosen are two studies that carried out microarray analysis on patient tissue samples from various stages of the oesophageal carcinogenic sequence. The Kimchi *et al* (2005) raw expression data was downloaded from the GEO database and the Ostrowski *et al* (2007) data was obtained directly from the authors (Kimchi *et al.*, 2005, Ostrowski *et al.*, 2007).

The Kimchi *et al* (2005) study examined gene expression data from 8 patients with oesophageal adenocarcinoma. There were three biopsies taken from each patient from normal squamous tissue, BO tissue and OAC tissue (Kimchi *et al.*, 2005). This study contained 30 normal squamous samples and 29 BO samples.

Both of these studies used tissue samples from patients with BO, however the group of patients involved in the Kimchi *et al* (2005) publication had developed BO associated OAC whereas the patients involved in the Ostrowski *et al* (2007) study had not developed OAC (Kimchi *et al.*, 2005, Ostrowski *et al.*, 2007). The raw data was subjected to the same gene funnelling analysis as previously described (Figure 3.2; transcripts marked 'absent' were removed; fold change > 1.5 and p-value < 0.05 were included). There are a number of genes with altered expression profiles that are specific to each study (Figure 3.2; Figure 3.7). There were a total of 3,011 genes with significant changes in expression from the Kimchi *et al* (2005) dataset with 1,548 of those genes specific to patients with BO associated adenocarcinoma (Kimchi *et al.*, 2005). The remaining 1,463 transcripts were

shared between both the Kimchi *et al* (2005) and Ostrowski *et al* (2007) studies with a further 2,354 genes altered in the Ostrowski *et al* (2007) study alone (Kimchi *et al.*, 2005, Ostrowski *et al.*, 2007). There were a total of 3,817 genes altered during BO development according to the Ostrowski *et al* (2007) study. The differences in the number of differentially expressed genes that were identified in both of these studies is due to the fact that the Kimchi *et al* (2005) dataset represents patients with BO and BO associated adenocarcinoma whereas the Ostrowski *et al* (2007) study examined patients with long segment BO only (Kimchi *et al.*, 2005, Ostrowski *et al.*, 2007). Also, the Ostrowski *et al* (2007) study has greater sample numbers and as such has increased statistical power to identify more subtle alterations in gene expression values (Ostrowski *et al.*, 2007).

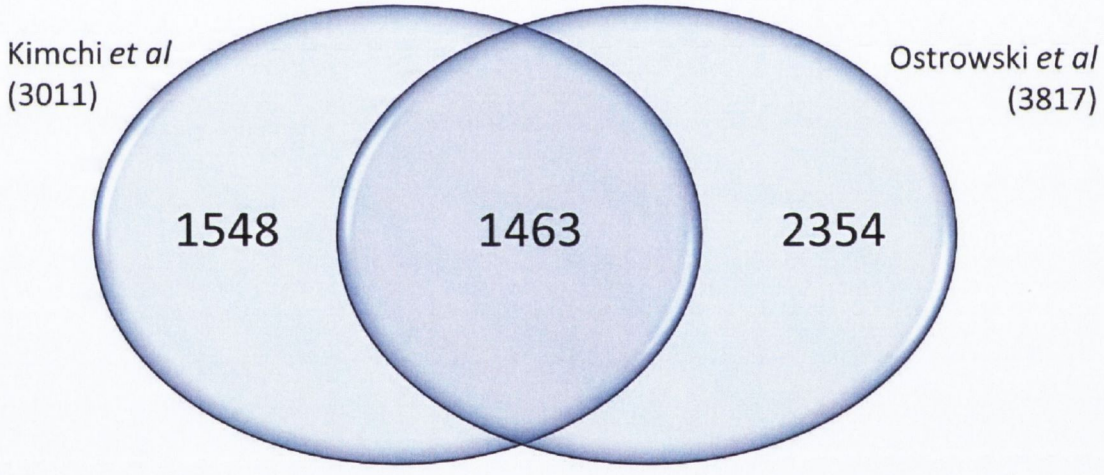


Figure 3.7: Venn diagram comparing gene lists of transcripts with altered expression profiles from both Kimchi *et al* (2005) and Ostrowski *et al* (2007) studies. Microarray studies examining alterations in gene expression profiles at various stages of the carcinogenic sequence were also used in this study. There were 1463 transcripts with altered expression shared between both studies. There were 1548 transcripts specific to the Kimchi *et al* (2005) study and 2354 transcripts specific to the Ostrowski *et al* (2007) study (Kimchi *et al.*, 2005, Ostrowski *et al.*, 2007).

Hierarchical clustering is a useful tool to visualise the degree of similarity between samples in a high density analysis study and are often used in conjunction with heat maps. Hierarchical clustering, utilising Euclidean distance analysis, and the gene list of

transcripts with statistically significant altered expression profiles from both patient studies is described in Figure 3.8. Conditional clustering of the genes with altered expression profiles from the Kimchi *et al* (2005) study demonstrated that one of the BO samples appears to be more similar to the OAC tissue than the other BO tissue samples (Kimchi *et al.*, 2005).

3.3.4 Identification of DCA responsive genes that are also altered during in progression through the oesophageal carcinogenic sequence

A comparative informatics strategy was developed to identify a subset of genes with altered expression profiles during oesophageal cancer development that also exhibited squamous and adenomatous cell type specific transcriptional responses to DCA. It is hypothesised that DCA may contribute to the development of oesophageal adenocarcinoma, therefore, microarray gene expression analysis was utilised to determine if DCA responsive genes were also altered in patient studies charting oesophageal carcinogenesis. These genes were identified by comparing two independent patient studies with gene expression data from a study of DCA regulated genes in two oesophageal cell lines (Ostrowski et al., 2007, Kimchi et al., 2005). This integration of independent microarray studies, including previously published data, is a comparative informatics approach that facilitated the extraction of clinically relevant information from an *in vitro* cell line study.

This study has previously identified a cohort of DCA responsive genes in two oesophageal cell lines (Het-1A - 7,667; SKGT4 - 4,520). Altered gene expression profiles during progression through the carcinogenic sequence have also been determined from two patient studies charting oesophageal carcinogenesis (Kimchi *et al* (2005) - 3011; Ostrowski *et al* (2007) - 3817) (Kimchi et al., 2005, Ostrowski et al., 2007).

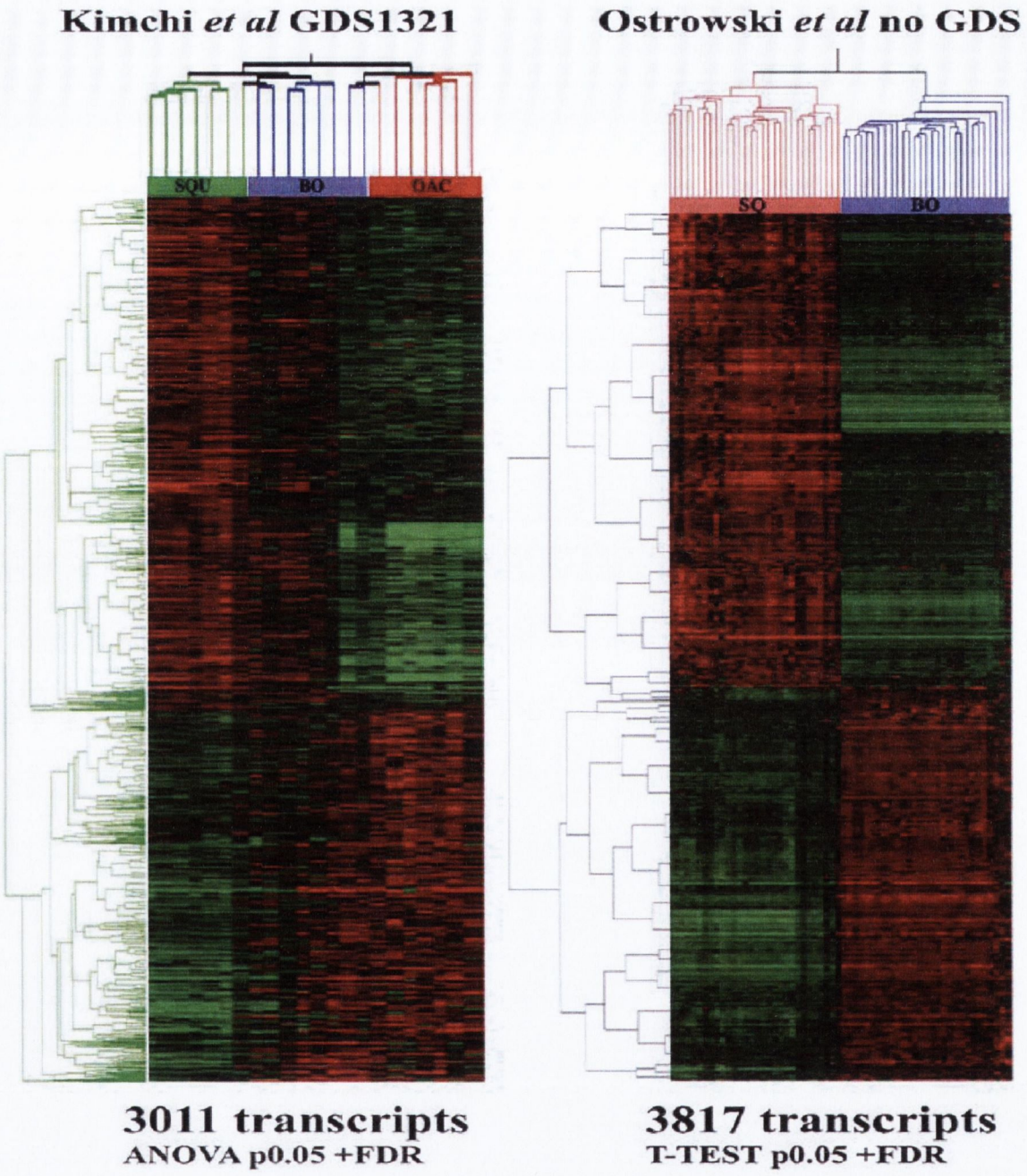


Figure 3.8: Unsupervised hierarchical clustering of transcripts with altered gene expression profiles in various stages of the oesophageal carcinogenic sequence. Gene expression levels at various stages of the oesophageal carcinogenic sequence were defined in both the Kimchi *et al* (2005) study and the Ostrowski *et al* (2007) study. Following gene funnelling of the microarray datasets as previously described (Figure 3.2), hierarchical and conditional clustering of resultant gene lists was carried out using Euclidean distance analysis. Differentially expressed genes in both studies successfully segregated each tissue type into distinct groups (Ostrowski et al., 2007, Kimchi et al., 2005).

In order to highlight genes with altered expression in BO that are important in bile acid signalling, the dataset of DCA responsive genes was compared to each of the patient

cohorts using the integration strategy illustrated in Figure 3.9 and Venn diagrams to compare each dataset. There were 954 DCA responsive genes identified in the BO samples from the Kimchi *et al* (2005) study. This represented 32% of the total number of differentially expressed genes in the Kimchi *et al* (2005) dataset following reanalysis of the raw data according to the strategy described in Figure 3.2 (Kimchi *et al.*, 2005). This subset of genes was comprised of genes specific to both the Het-1A cells (495 genes; 16%) and the SKGT4 cells (257 genes; 9%). There were also 202 genes (7%) that were shared between both cell lines and were altered during progression through the carcinogenic sequence. In relation to the Ostrowski *et al* (2007) study, 37% of the genes with altered expression in the development of BO were DCA responsive genes (Ostrowski *et al.*, 2007). This included 677 genes (18%) that were Het-1A specific, 387 genes (10%) that were SKGT4 specific and 354 genes (9%) that were DCA responsive in both cell lines (Figure 3.10).

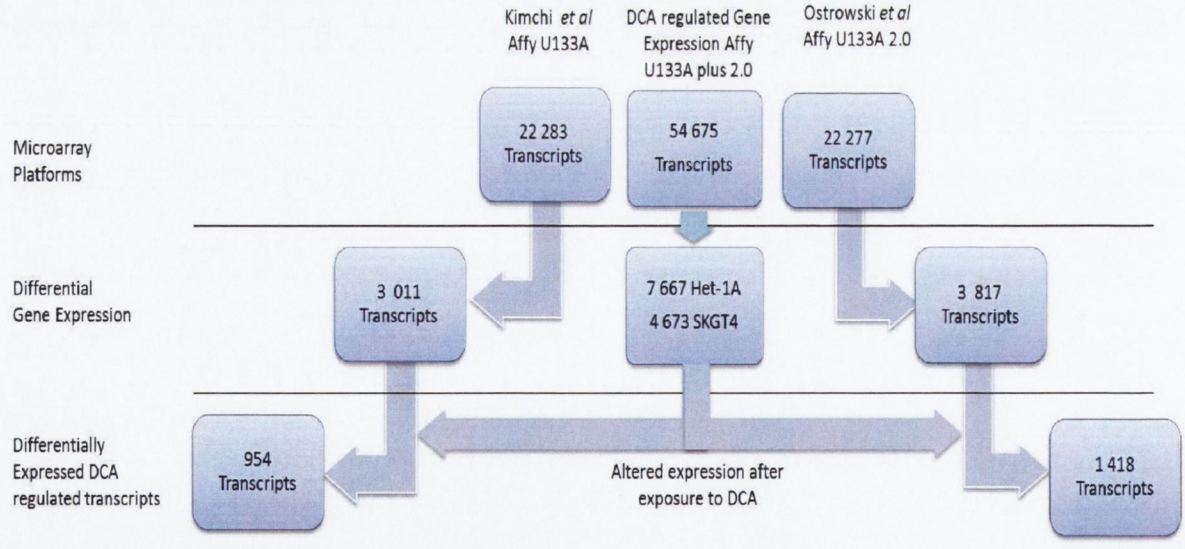


Figure 3.9: Flow diagram illustrating strategy by which DCA responsive genes were integrated into microarray datasets charting oesophageal carcinogenesis. The analysis strategy described in Figure 3.2 was carried out on each microarray dataset individually to establish a set of differentially expressed genes in each study. A cohort of DCA responsive genes in Het-1A and SKGT4 cells was compared to the significantly altered genes from both the Kimchi *et al* (2005) and Ostrowski *et al* (2007) datasets. From a total of 3011 significantly altered genes in the Kimchi *et al* (2005) study, 954 were found to be DCA responsive. In the Ostrowski *et al* (2007) study, 1,418 of the 3,817 altered genes were found to be DCA responsive (Kimchi et al., 2005, Ostrowski et al., 2007).

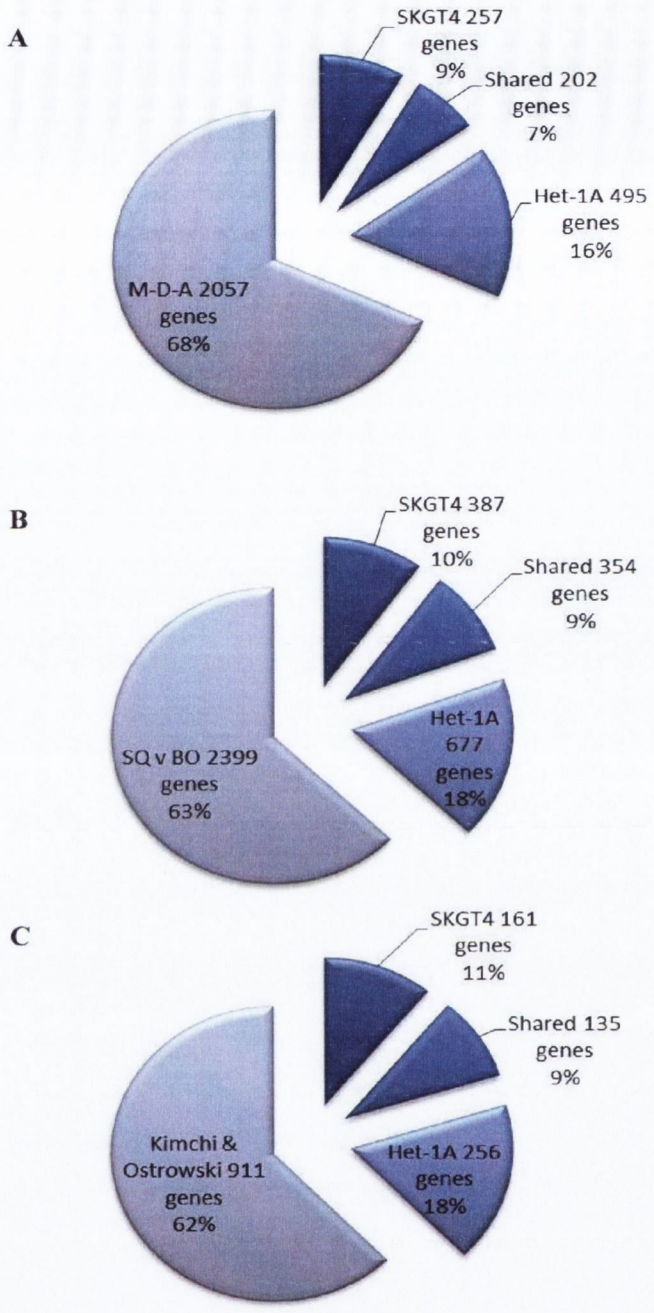


Figure 3.10: Distribution of transcripts with altered gene expression profiles during oesophageal adenocarcinogenesis that are DCA responsive. Significantly altered transcripts from the oesophageal carcinogenic sequence were compared to DCA responsive genes. (A) The Kimchi *et al* (2005) study included samples from normal, BO and OAC tissue which contained 954 DCA responsive genes (32%) that were differentially expressed. The DCA responsive genes contained 495 (16%) Het-1A specific genes, 257 (9%) SKGT4 specific gene and 202 (7%) DCA responsive genes were shared between both cell lines (Kimchi et al., 2005). (B) The Ostrowski *et al* (2007) study contained 1034 DCA responsive genes (37%) with altered expression profiles between normal and BO tissue samples. These DCA responsive genes contained 677 (18%) Het-1A specific genes, 387 (10%) SKGT4 specific genes and 354 (9%) genes were shared between both cell lines (Ostrowski et al., 2007). (C) All three microarray studies combined together demonstrated that 38% of transcripts with altered expression in both the Kimchi *et al* (2005) and Ostrowski *et al* (2007) studies are regulated by DCA (Kimchi et al., 2005, Ostrowski et al., 2007).

The top 100 DCA responsive genes from each cell line in the Ostrowski *et al* (2007) study are illustrated on a heat map in Figure 3.11 (Ostrowski et al., 2007). This selection of DCA responsive transcripts included genes that were both induced and repressed in response to DCA exposure in both the Het-1A (Figure 3.11A) and SKGT4 (Figure 3.11B) cell lines. The top network generated in the Ingenuity Pathways Analysis (IPA) software using the top 100 Het-1A specific DCA responsive genes with differential expression during BO development (Ostrowski *et al.*, 2007) is illustrated in Figure 3.12A. This network is involved in processes such as cellular movement and cellular morphology. Tribbles homolog 3 (TRB3) and RAR-related orphan receptor α (RORα) are two genes from this cohort central to this network. Both of these genes are upregulated in Het-1A cells alone in response to DCA exposure. Interestingly, the expression profiles of these genes in the microarray analysis of patients with BO associated adenocarcinoma (Kimchi *et al.*, 2005) demonstrate that expression of TRB3 and RORα is significantly lower in adenocarcinoma tissue than normal tissue (Figure 3.12 B; p-value < 0.05). TRB3 and RORα both interact with NF-κB and as such may alter this signalling pathway. The top network associated with the top 100 SKGT4 specific DCA responsive genes with differential expression during BO development (Ostrowski *et al.*, 2007) is illustrated in Figure 3.13A. This network is involved in cell-to-cell signalling. Both serpin pepidase inhibitor, clade A (alph-1 antiproteinase, antitrypsin), member 1 (SERPINA1) and prostaglandin E receptor 4 (subtype EP4) (PTGER4) are down regulated in response to DCA in an oesophageal adenocarcinoma cell line (SKGT4). Also, both of these molecules interact with the extracellular signal-regulated kinases (ERK) 1/2 dimer. In the adenocarcinoma microarray gene expression study, both SERPINA1 and PTGER4 display increased expression levels in BO and OAC patient tissue samples (Figure 3.13B).

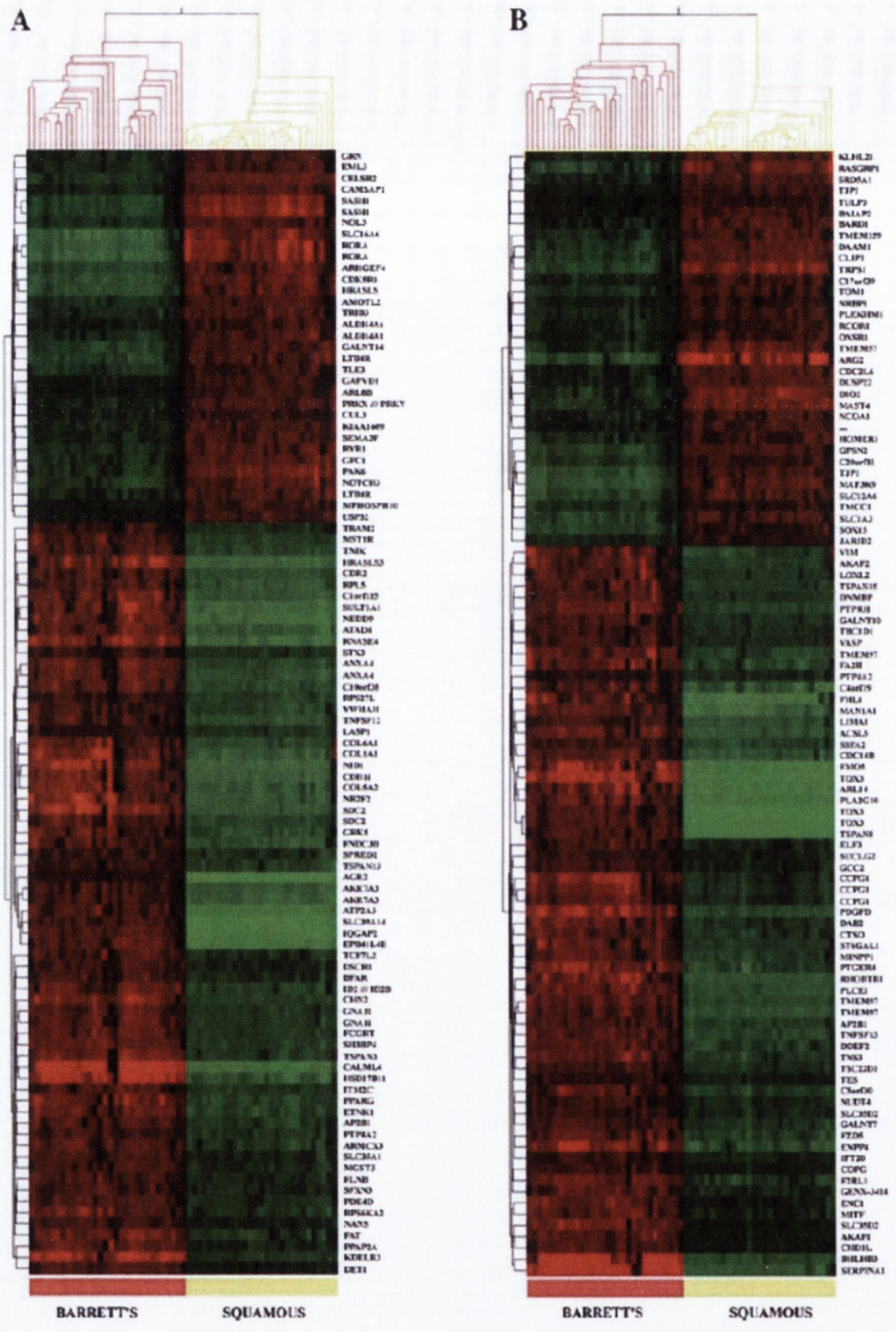


Figure 3.11: Heat map of DCA responsive genes with significantly altered gene expression profiles in the Ostrowski *et al* (2007) study. DCA responsive genes in (A) Het-1A and (B) SKGT4 cells were also significantly altered between squamous and BO patient tissue. The top 100 transcripts were clustered using unsupervised hierarchical clustering. The expression profiles of these DCA responsive genes were capable of grouping all squamous and BO sample together as two distinct groups (Ostrowski et al., 2007).

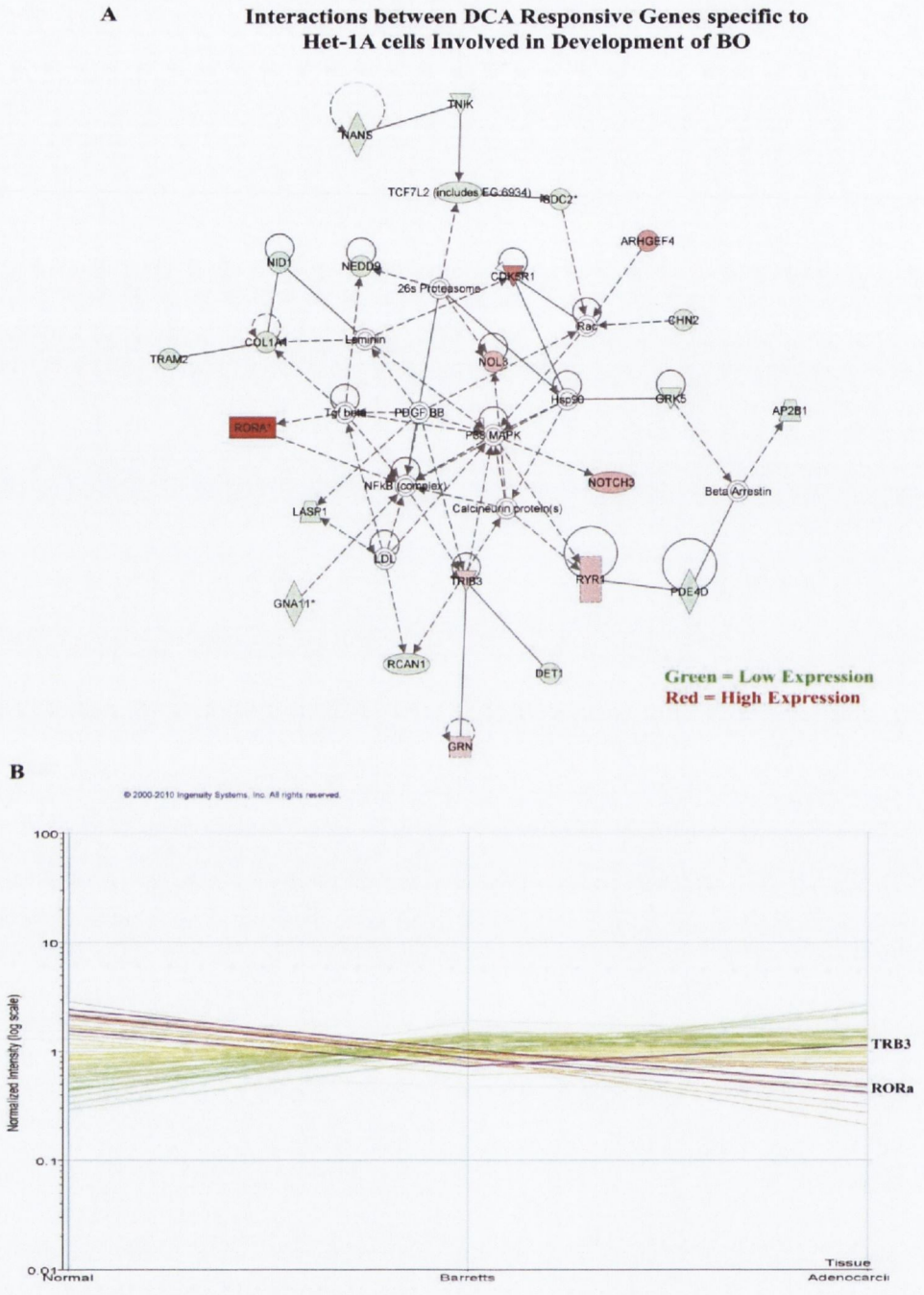


Figure 3.12: Network analysis & expression data in oesophageal carcinogenic sequence of top 100 Het-1A specific DCA regulated genes with differential expression during BO development. IPA analysis of the top 100 Het-1A specific DCA regulated genes with differential expression during BO development in the Ostrowski *et al* (2007) study was carried out (Ostrowski et al., 2007). (A) The top network that was associated with this dataset was involved in processes such as cellular movement and cellular morphology. TRB3 is induced in Het-1A cells in response to DCA and is central to this network (red - high expression; green - low expression). (B) Expression profiles of the members of this network in the oesophageal adenocarcinoma dataset (Kimchi *et al.*, 2005) demonstrated that both TRB3 and RORα, which are induced by DCA in Het-1A cells specifically, exhibit significantly decreased expression levels in BO and OAC samples compared to normal tissue (highlighted by purple line; p-value < 0.05). There are two transcripts targeting RORα expression on the microarray.

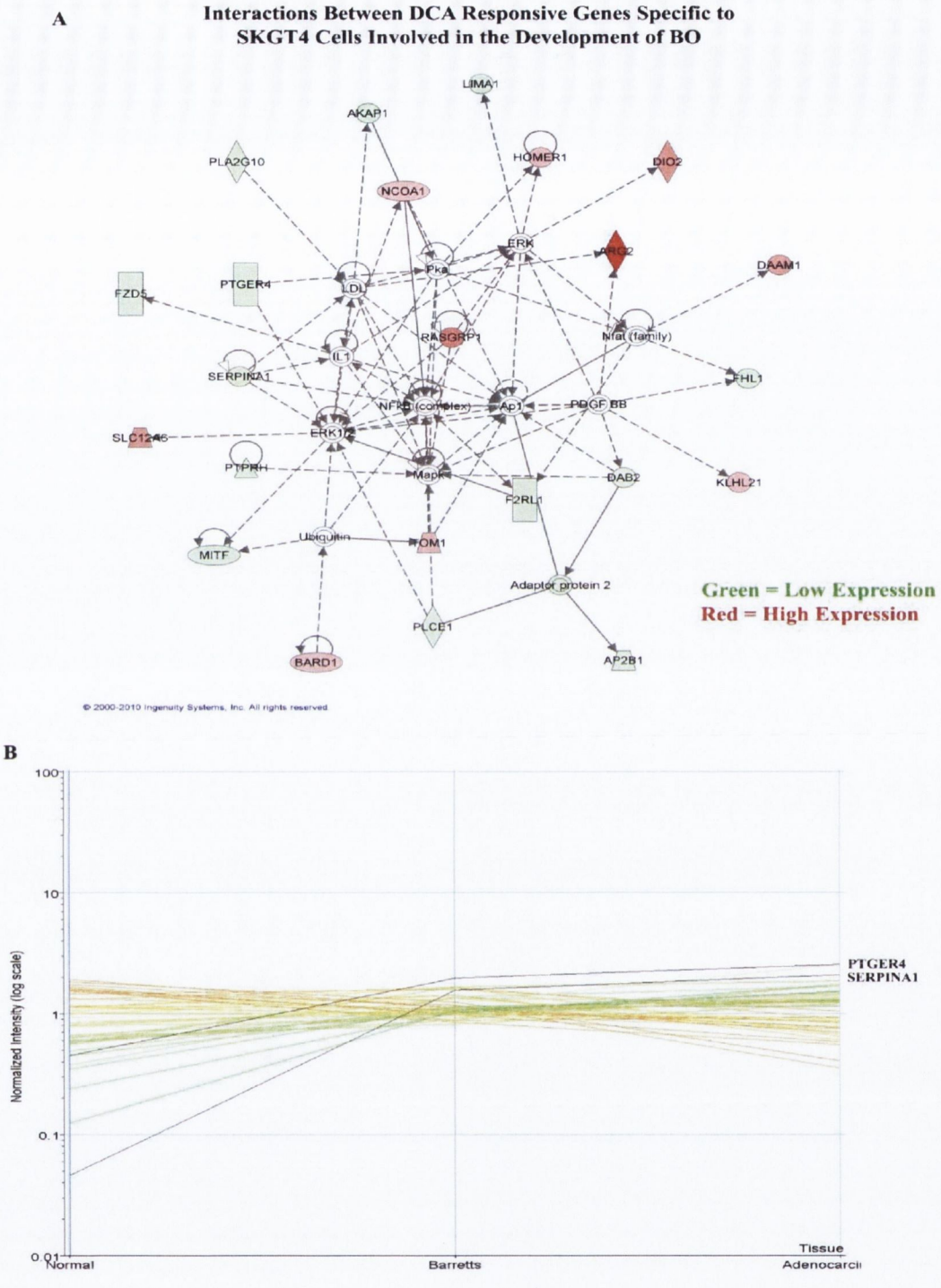


Figure 3.13: Network analysis & expression data in oesophageal carcinogenic sequence of the top 100 SKGT4 specific DCA regulated genes with differential expression during BO development. IPA analysis of the top 100 SKGT4 specific DCA regulated genes with differential expression during BO development in the Ostrowski *et al* (2007) study was carried out (Ostrowski et al., 2007). (A) The top network that was associated with this dataset was involved in cell-to-cell signalling. SERPINA1 and PTGER4 are repressed in SKGT4 cells specifically in response to DCA (red - high expression; green - low expression). (B) Expression profiles of the members of this network in the oesophageal adenocarcinoma dataset (Kimchi *et al*., 2005) demonstrated that both SERPIN1A and PTGER4, which are induced by DCA in SKGT4 cells specifically, exhibit significantly increased expression levels in BO and OAC samples compared to normal tissue (highlighted by purple line; p-value < 0.05).

3.3.5 Systems biology analysis identifies cellular processes that are associated with DCA regulated genes with altered gene expression during oesophageal carcinogenesis

A systems biology approach was implemented to identify relationships, mechanisms, functions and pathways of relevance to the genelists derived from the microarray analysis. Ingenuity Pathways Analysis (IPA) software contains a database of annotated cellular processes and pathways. This program uses powerful biological filters that focus on the cellular pathways and processes that are most relevant to the experimental model that is under investigation. Datasets containing DCA regulated genes, genes involved in adenocarcinogenesis and also genes that are both DCA responsive and altered during adenocarcinogenesis have been established. Each of these datasets were analysed using the IPA software to confer functional significance on each cohort and identify potential mechanisms through which bile acids may promote oesophageal carcinogenesis.

IPA software was used to create gene networks centred on a gene of interested. TRB3 has been identified as induced in response to DCA in the Het-1A cells alone and has also been identified as a gene that is lost during cancer development (Figure 3.12). A network illustrating the molecular interactions of TRB3 was created using IPA (Figure 3.14). The expression levels of components of this network are based on the expression levels of the genes in Het-1A cells exposed to DCA. This network illustrates that TRB3 is upregulated in response to DCA exposure of the Het-1A cells. TRB3 interacts with a number of other molecules with altered expression in Het-1A cells in response to DCA. Interestingly, TRB3 also interacts with a number of molecules that are regulated by DCA in the SKGT4 cells specifically, possibly as a result of loss of TRB3 expression (Figure 3.15).

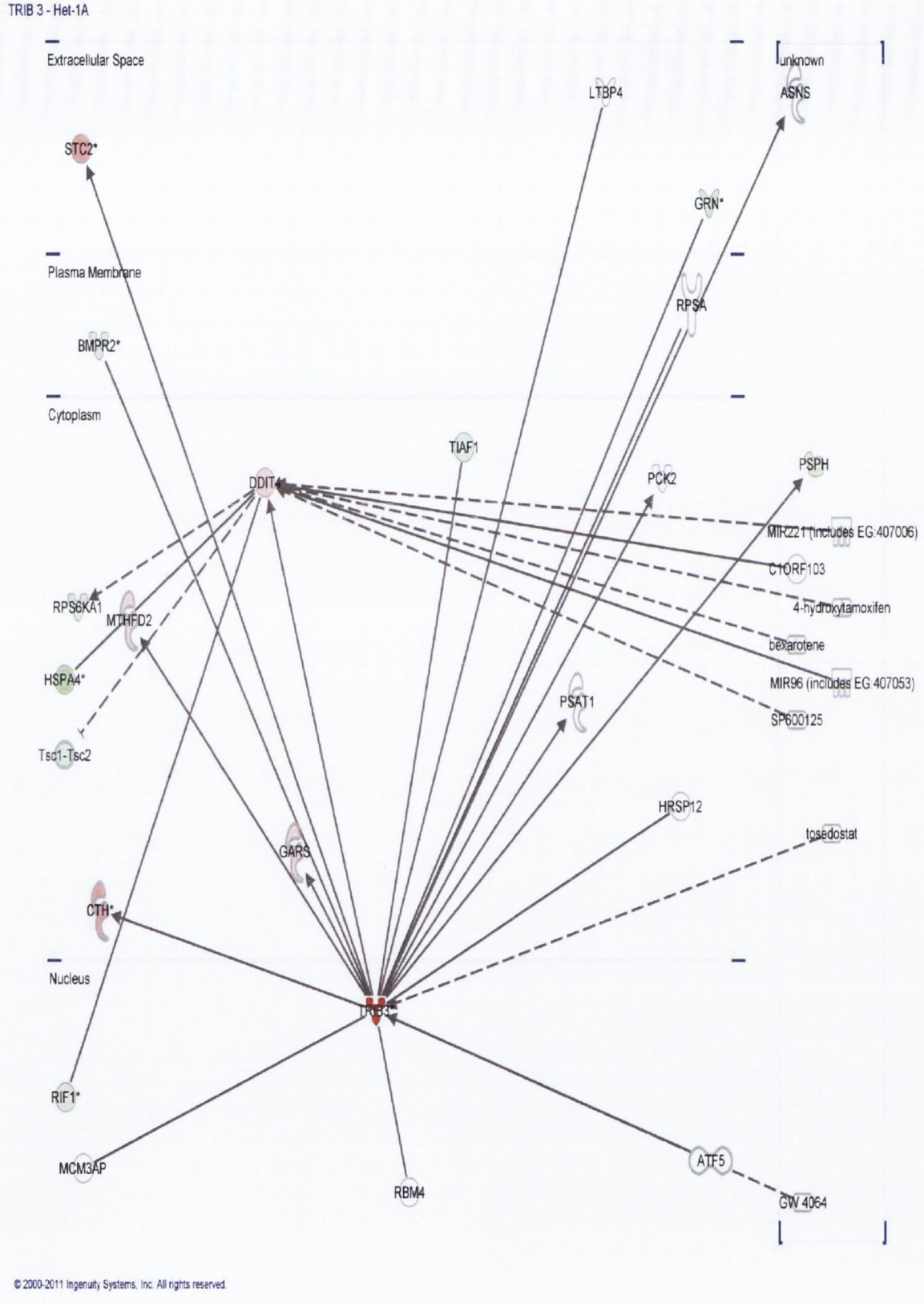


Figure 3.14: Gene network containing Het-1A specific responses to DCA. IPA was utilised to create a gene network composed of DCA responsive genes specific to the Het-1A cell line. TRB3 was the central node of this gene network and is up regulated in this cell in response to DCA exposure (indicated by the red colour). Coloured symbols indicate molecules present in a gene list comprised of DCA responsive gene specifically altered in Het-1A cells alone (green represents loss of expression, red represents induction of expression following DCA treatment).

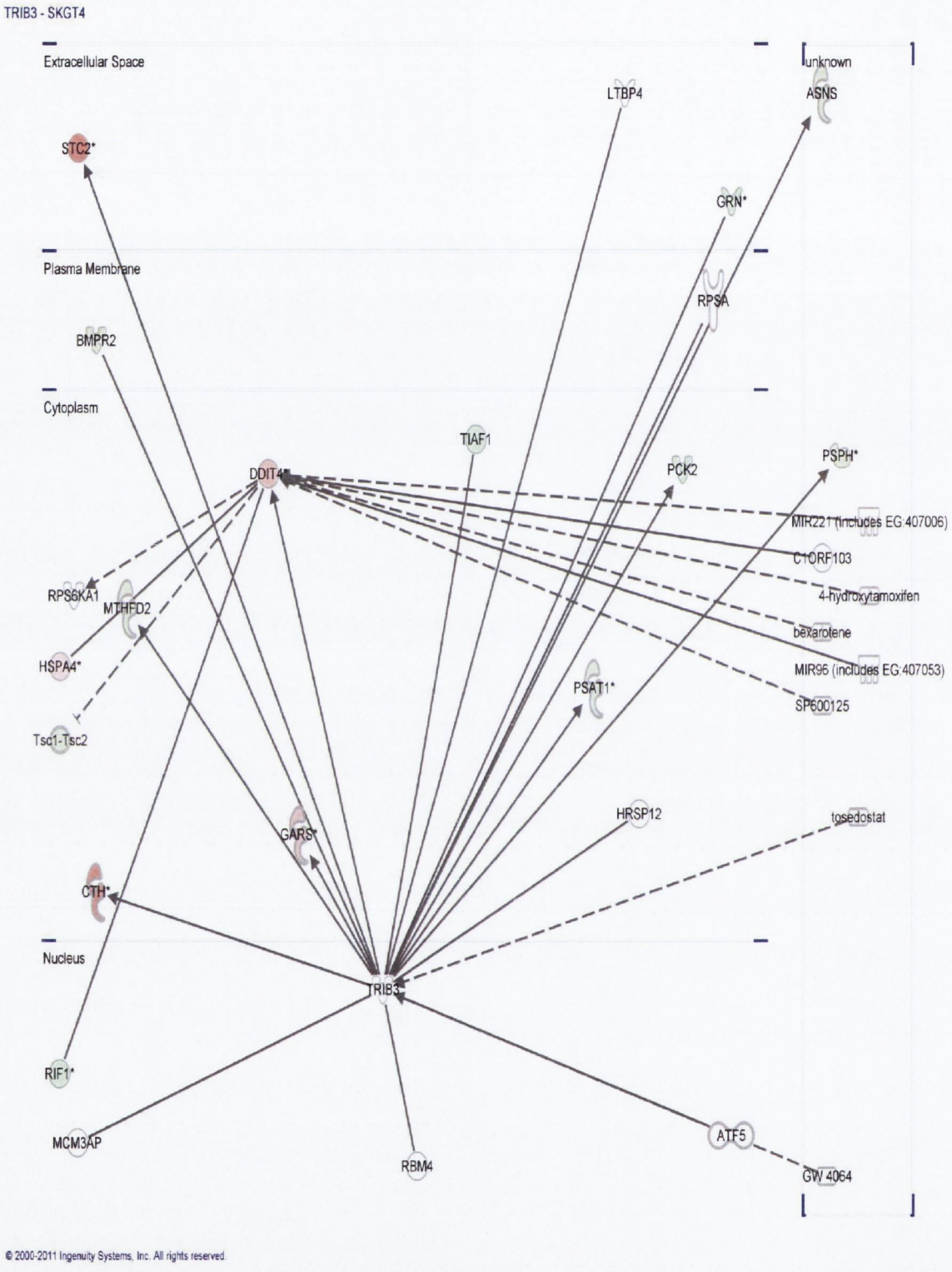


Figure 3.15: Gene network containing SKGT4 specific responses to DCA. IPA was utilised to create a gene network composed of DCA responsive genes specific to the SKGT4 cell line. While TRB3 is a central node in this network, this gene is induced by DCA in the Het-1A cells alone, hence the lack of colour in this diagram. TRB3 does interact with a number of genes that are induced by DCA in this cell line alone. Coloured symbols indicate molecules present in a gene list comprised of DCA responsive gene specifically altered in SKGT4 cells alone (green represents loss of expression, red represents induction of expression following DCA treatment).

IPA software was also used to identify cellular processes and pathways that were linked to differentially expressed genes identified in this study. The cohort of all DCA responsive genes is associated with cancer related processes, gastrointestinal disease, cell cycle, cell death and cellular growth & proliferation (Figure 3.16 A). This suggests a role for DCA regulation in cancer related processes prior to the inclusion of oesophageal patient data. Additionally, subsequent integration of oesophageal patient data with the bile acid expression data identified cancer related processes as the principal cellular process associated with this dataset (Figure 3.16 B&C) (Kimchi et al., 2005, Ostrowski et al., 2007). Cell death and cellular growth & proliferation are associated with SKGT4 specific transcriptional responses to DCA that are also involved in adenocarcinoma development. These data indicate that DCA may contribute to the underlying molecular mechanisms that are required for cancer development in the oesophagus by affecting cell death and cell proliferation. Other processes identified which may be associated with cancer development included cellular movement, cell to cell signalling & interaction and immune response. These data indicate a number of mechanisms by which bile acids may contribute to oesophageal cancer development.

3.4 Discussion

The development of oesophageal cancer involves the accumulation of mutations in cancer cells that confer a competitive advantage over normal cells (Maley, 2007). A central role for bile acids in progression through the oesophageal carcinogenic sequence has been hypothesised (Souza *et al.*, 2008). Therefore, expression analysis was carried out to identify genes with altered expression in BO and OAC development that are also transcriptional regulated by bile acids. A comparative bioinformatics strategy was developed and successfully implemented.

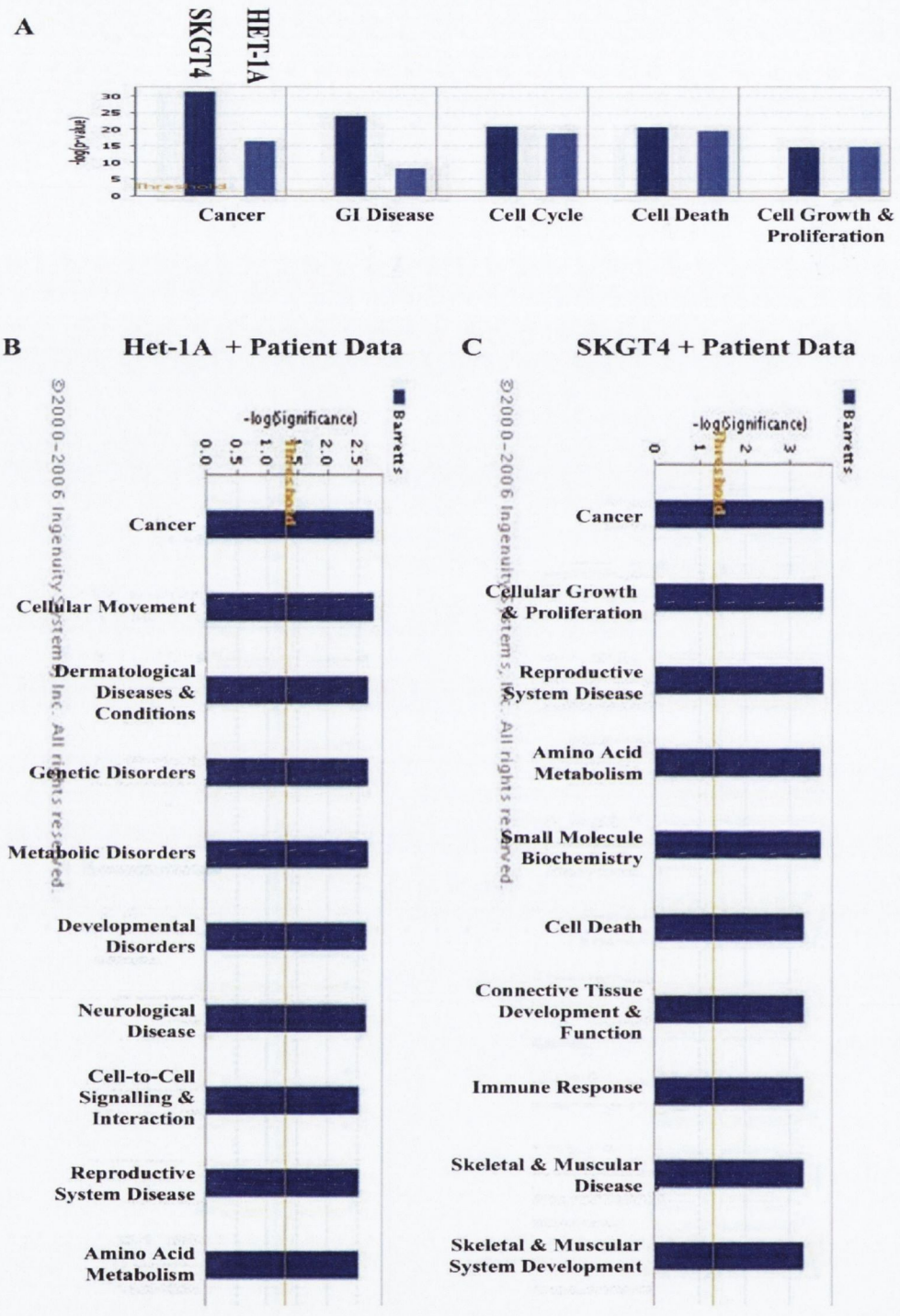


Figure 3.16: Top ranked cellular processes linked with a cohort of DCA responsive genes with altered gene expression profiles in oesophageal adenocarcinogenesis. IPA software determines cellular processes and pathways that are linked to specific datasets. (A) DCA responsive genes identified in both Het-1A and SKGT4 cell lines were linked with processes such as cancer, cell death and cellular growth & proliferation. (B) Cancer related processes are also linked with a cohort of DCA responsive genes in Het-1A cells with altered expression during oesophageal adenocarcinogenesis. (C) DCA regulated genes in SKGT4 cells with altered expression in patient data were linked with cancer, cell death and cellular growth & proliferation (Kimchi et al., 2005, Ostrowski et al., 2007). (Threshold was set based on a significance value of 0.05.)

This strategy determined that approximately one third of genes that are differentially expressed during oesophageal adenocarcinoma development in two independent patient studies are regulated by DCA (Kimchi et al., 2005, Ostrowski et al., 2007). These data provide further evidence for the involvement of bile acids in the development of oesophageal adenocarcinoma. Cellular processes and pathways through which bile acids may act to contribute to cancer development have also been proposed. Approximately one third of these genes are involved in the cellular response to stress.

Gene expression profiling using microarray technology is an extremely effective strategy in the investigation of cellular biology and identification of the underlying mechanisms of human diseases. Gene expression studies generate vast quantities of data, the majority of which remains largely unused in the original study. The remaining data may not be required for the original study, however, these data may be extremely useful in the context of other similar investigations. The Gene Expression Omnibus (GEO) repository is a central source of archived raw data from microarray gene expression studies. As microarray experiments are expensive and not readily available in a routine laboratory environment, resources such as the GEO database are an invaluable tool to extract maximum benefit and impact from the original study by enhancing future research. A search of the GEO database using the keywords 'esophageal' or 'Barrett's' found 59 and 24 related studies respectively. This study successfully utilised the GEO database to enhance and expand on gene expression analysis that had been previously carried out in the laboratory. The combination of an original gene expression analysis (Duggan et al., 2010) supplemented with a previously published gene expression data facilitated the execution of a comparative study that integrated both datasets (Kimchi et al., 2005). This comparative strategy was also carried out on a previously published microarray dataset that was obtained directly from the authors of a BO patient study (Ostrowski et al., 2007).

Two previously published patient studies charting oesophageal carcinogenesis were integrated with an original DCA responsive dataset that was generated in this study (Duggan et al., 2010, Kimchi et al., 2005, Ostrowski et al., 2007). Both patient studies were reanalysed using the strategy that was developed for this study. The original analysis of the Kimchi *et al* (2005) data utilised a more stringent analysis strategy to determine genes with significant alterations in gene expression profiles in the patient data set. This resulted in the identification of a much smaller cohort of genes with statistically significant alterations in gene expression than the cohort identified in the reanalysis of the data (Kimchi et al., 2005). The less stringent analysis used here was more suited to the overall aim of this study which was to integrate a number of microarray gene expression studies. Both analysis strategies identified similar pathways and processes however the reanalysis strategy identified a larger cohort of genes which were still significantly altered. The original publication of this microarray data concentrated on this small cohort of differentially expressed genes (214) and concluded that OAC development is associated with suppression of genes involved in epidermal differentiation. Hierarchical clustering successfully segregated the three tissues types using this cohort of 214 differentially expressed genes (Kimchi *et al.*, 2005). The Kimchi *et al* (2005) study only considered genes that were up or down regulated from normal to BO and again up or down regulated from BO to OAC gene profiles as significant (Kimchi *et al.*, 2005). In contrast, the reanalysis strategy developed considered all combinations of up and down regulation as significant even those where a gene was only altered between two of the three conditions. The microarray gene expression analysis strategy that was developed and implemented for the reanalysis of the Kimchi *et al* (2005) dataset in this study chose a significance level that provided the most comprehensive genelists for comparison with other datasets.

The Ostrowski *et al* (2007) study analysed oesophageal squamous epithelium (n=30) and Barrett's metaplasia (n=29) samples in patients with long segment BO. The reanalysis strategy that was developed and implemented on the microarray datasets in this study yielded a similar number of differentially expressed genes as the original published analysis. The Ostrowski *et al* (2007) study concluded that BO develops in response to exposure to harmful elements of refluxate. The authors propose that BO develops as a consequence of morphological and physiological changes that occur to create a mucosa that is more resistant to exogenous agents contained in refluxate (Ostrowski *et al.*, 2007).

The main objective of this study was to identify genes displaying differential expression patterns during development of adenocarcinoma that also demonstrated cell type specific responses to bile acid exposure. Differentially expressed transcripts from two patient studies were integrated with the cohort of DCA responsive genes in order to extract a subset of bile acid regulated genes that were also involved in adenocarcinoma development (Kimchi et al., 2005). A limitation of this comparative strategy is that all three studies were performed using different microarray formats. In order to overcome this, all data was converted into nomenclature as defined by the U133A microarray format (Affymetrix). While these data conversions facilitated the integration of the three studies, it also resulted in loss of a number of genes that were present on the larger microarray formats. The Affymetrix U133A 2.0 and U133A plus 2.0 formats contain 22,277 and 54,675 transcripts respectively. The Affymetrix U133A microarray contains 22,283 which equates to a loss of over half the transcripts on this format.

When the inter-comparison of these studies was completed, it was observed that approximately one third of transcripts with altered expression profiles in each of patient studies were also DCA responsive. This may suggest a role for DCA in progression

through the oesophageal adenocarcinoma sequence possibly as a result of the induction of cellular stress. DCA responsive genes specific to Het-1A cells with altered expression patterns in oesophageal disease include the clock gene RAR-related orphan receptor α (RORα) and also TRB3. DCA responsive genes specific to SKGT4 cells with altered expression patterns in oesophageal disease include SERPINA1 and PTGER4.

TRB3 is interesting as this gene has an array of binding partners such as mitogen-activated protein kinase family members and AKT (Figure 3.14A) and also interacts with NF-κB (Figure 3.12). TRB3 interaction with NF-κB is interesting as NF-κB signalling has been demonstrated to be deregulated during Barrett's carcinogenesis (Abdel-Latif *et al.*, 2009). This microarray gene expression analysis determined that TRB3 is downregulated during oesophageal adenocarcinoma development (Figure 3.12 B). Of note, TRB3 was only induced by DCA in Het-1A cells (Figure 3.14), and not in SKGT4 cells (Figure 3.15). Loss of TRB3 expression during adenocarcinoma development and loss of TRB3 induction by DCA in an adenocarcinoma cell line is an interesting finding. Network analysis has demonstrated that loss of TRB3 expression results in substantial alterations in the expression levels of downstream molecules (Figure 3.15). These gene expression alterations are specific to the adenocarcinoma cell line and as such may provide insights into the contribution of bile acid signalling to oesophageal adenocarcinoma development. This level of transcriptional response could have far reaching consequences and requires further investigation. TRB3 functionality was further investigated and published by this group (Duggan *et al.*, 2010). Further evidence as to the importance of aberrant TRB3 expression was uncovered in this laboratory. It has been demonstrated that increased levels of IL8 and IL6 are observed when TRB3 is suppressed in Het-1A cells. TRB3 re-expression in SKGT4 cells resulted in a decrease in the level of IL8 & IL6 expression. This implies that TRB3 has an important role to play in the regulation of inflammatory

responses in oesophageal cells. IL8 and IL6 are inflammatory cytokines and loss of TRB3 expression in BO and OAC may contribute to the increased levels of inflammation that is associated with oesophageal adenocarcinogenesis (Duggan *et al.*, 2010, Fitzgerald *et al.*, 2002). Furthermore, NF-κB signalling is involved in a number of cellular processes. As such loss of TRB3 interaction during development of OAC is extremely interesting and requires further investigation. Loss of TRB3 induction by SKGT4 adenocarcinoma cells may be a result of an adaptive mechanism of cancer cells to develop resistance to DCA regulation. This further illustrates the potential of gene expression studies to generate more data than is relevant to a single study and highlights the importance of collaboration and cooperation to extract maximum impact from each experiment that is carried out.

The ERK1/2 dimer is central to a network involved in cell-to-cell interaction which is associated with the top 100 SKGT4 specific DCA regulated expression with differential expression during BO development. ERK has previously been demonstrated to be involved in DCA signalling (Looby *et al.*, 2009). Network analysis has demonstrated that two genes repressed in response to DCA exposure of an oesophageal adenocarcinoma cell line, SKGT4 (Figure 3.13). Both SERPINA1 and PTGER4 interact with the ERK1/2 dimer. These data provide further evidence that bile acid signalling can occur via ERK1/2.

These findings have demonstrated that the comparative analysis strategy that was developed in this study is a powerful tool linking *in vitro* and *in vivo* data to create a more comprehensive overview of bile acid signalling in the context of oesophageal adenocarcinoma development.

Unsupervised hierarchical clustering using a list of DCA responsive genes resulted in the segregation of each stage of oesophageal adenocarcinoma disease progression in both

patient microarray studies (Kimchi et al., 2005, Ostrowski et al., 2007). The segregation of patient samples based on disease stage characterised using phenotypic diagnostic markers is maintained using only the gene expression profiles of DCA responsive genes in a disease setting. In the case of breast cancer, gene expression profiling has been utilised as a method to classify tumours and also to predict responses of individual tumours to therapy (Weigelt *et al.*, 2010). A similar approach is being developed in relation to gene expression profiles at the different stages of the oeosphageal carcinogenic sequence (Ito *et al.*, 2007). This study has demonstrated that a cohort of DCA responsive genes can classify disease tissue types successfully. Further investigations to define a diagnostic gene expression signature may include differential gene expression in response to DCA exposure.

Also of note in the hierarchical conditional clustering carried out using DCA responsive genes of the Kimchi *et al* (2005) patient cohort was that one BO sample appeared to correlate more with the OAC tissue samples than the other BO samples (Kimchi *et al.*, 2005). This may have occurred because the biopsy was contaminated with a section of adenomatous tissue or as a result of a field effect from nearby adenomatous tissue. Alternatively, this BO biopsy may represent an intermediate lesion that is potentially an area of dysplasia. Further investigations using this BO sample as an intermediate stage between BO and OAC could provide further insight into progression through the carcinogenic sequence. This abnormal appearance of this sample may also be as a result of a field effect from nearby OAC tissue. Cancer is thought to develop with the progressive transformation of cells facilitating the lateral spreading of the tumour (Yakoub et al., 2010).

In order to establish the functional relevance of DCA responsive genes with altered gene expression profiles in oesophageal carcinogenesis, a systems biology analysis approach was applied. Analysis of cellular processes and pathway connected to this cohort of genes was carried out using Ingenuity Pathways Analysis (IPA) software. This software identifies cellular functions, canonical pathways and networks of genes involving members of the cohort under investigation. Analysis of this cohort of DCA regulated genes involved in progression through the oesophageal carcinogenic sequence identified cancer, gastrointestinal disease, cell death and cell growth & proliferation as processes with a significant links to this cohort of genes (p-value < 0.05, Fisher's Exact t-test). The involvement of bile acids in oesophageal adenocarcinoma has been postulated. Higher bile acid concentrations have been detected in oesophagitis and BO patients (Nehra *et al.*, 1999). However, this is merely a correlation and does not suggest the mechanisms by which bile acids may be contributing to oesophageal adenocarcinogenesis. Previous investigations have often been limited to assessing one cellular mechanism by which bile acids may contribute to adenocarcinoma in publication. While this strategy has uncovered a number of relevant mechanisms such as DNA damage (Zhang et al., 2009a) and gene regulation in response to bile acid (Burnat *et al.*, 2007, Dvorak *et al.*, 2007), this present study has described numerous genes, pathways and processes that demonstrate potential avenues through which bile acids may function in adenocarcinoma development (Figure 3.16). Further investigation is required to confirm the involvement of bile acids in the promotion of oesophageal adenocarcinoma. This study is an initial step towards a greater insight into the role of bile acids in oesophageal adenocarcinoma. A number of candidate cellular pathways and processes have been identified that may contribute to the development of oesophageal adenocarcinoma as a result of exposure to bile acids.

This study has utilised for the first time a comparative bioinformatics approach to assess the contribution of DCA responsive genes to the development of oesophageal diseases such as BO and adenocarcinoma. Pathways and processes such as cell death and cell growth & proliferation have been identified as the potential mechanisms that facilitate bile acid promotion of oesophageal disease. These processes will be investigated further in an attempt to identify the specific cellular mechanisms that bile acids may regulate to drive the development of adenocarcinoma.

Chapter 4

Results

The Identification of Novel Therapeutic Targets for the Treatment of Oesophageal Cancer using a Semi-automated High Throughput siRNA Library Screening

4 Development of a high throughput siRNA library screening strategy

4.1 Introduction

The introduction of RNA interference (RNAi) technology as a molecular tool has revolutionised cancer biology research and has had a profound effect on molecular biology (Editorial Nature Cell Biology, 2003) RNAi is a naturally occurring mechanism where small or short interfering RNA (siRNA) molecules target the mRNA of a gene of interest using sequence homology. This resulting double stranded RNA is then degraded by intracellular immune complexes originally defined as an anti-viral defence mechanism. RNAi as a research strategy is now commercially available and has become a useful tool to assess gene function (Krausz, 2007). The availability of libraries of siRNA in excess of 10,000 targets, advances in automated fluorescent microscopy and the development of liquid handling robots has led to a vast number of studies incorporating high throughput/high content siRNA library screens. The siRNA libraries are designed to target families and classes of genes such as G-protein coupled receptors (GPCRs), kinases and the druggable genome (set of genes coding for proteins that are capable of binding drug-like molecules),

RNAi screening aims to specifically inhibit one gene per well in order to phenotypically characterise the cell line under examination following loss of function of the target gene. RNAi screening has been implemented in a number of studies including the identification of novel genes involved in cell signalling pathways (MacKeigan *et al.*, 2005) and synthetic lethal screens to identify genes that sensitise cells to chemotherapeutic drugs (Turner *et al.*, 2008, Whitehurst *et al.*, 2007). RNAi screening is an extremely powerful tool; however caution is required at the experimental control and design stage of each study. The publication of badly designed studies containing unvalidated or inaccurate data may lead to a lack of confidence in this emerging technology.

High throughput siRNA screening involves the systematic testing of a significant number of targets in a single experiment. Risks are associated with such studies including the identification of false negative or false positive targets which can be a result of the quality of the reagents or the sensitivity of the assay (Echeverri *et al.*, 2006). Chemical modifications have been added to the design of siRNA molecules in an attempt to reduce off-targets effects of the experimental process (Jackson *et al.*, 2006). Recent advances have decreased the number of off-target effects associated with siRNA inhibition without completely eliminating them. Verification and validation experiments are an integral element of the siRNA screening process. Selection of appropriate controls relevant to each assay is also of extreme importance. Commercially available controls include non-targeting or scrambled siRNA molecules which do not target any known transcripts in the target genome. Despite the design of these negative controls, it is still possible that an off-target effect will be observed with these controls. It is important in the selection of negative controls that the resulting phenotype is as close to the baseline, untreated cells as possible (Echeverri and Perrimon, 2006). The selection of a known member of the pathway/process under examination is also recommended. An appropriate positive control will act as quality control for the screen and the efficiency of transfection. Stringent predefined values should be set for all controls both positive and negative to assess the quality of the screening process and in turn the validity of the results (Krausz, 2007).

The process of siRNA transfection requires a number of steps and reagents which need to be optimised for each cell line under examination. Transfection protocol can be based around a lipid transfection system (e.g. DharmaFECT reagent, Dharmacon; Lipofectamine 2000, Invitrogen), modifications on the siRNA molecules themselves (e.g. Accell siRNA, Dharmacon) or electroporation protocols for primary cells and difficult to transfect cell lines (Krausz, 2007). Previous siRNA transfections that had been completed by this group

in similar cell lines utilised a lipid based siRNA transfection protocol with DharmaFECT siRNA transfection reagent (Duggan *et al.*, 2010). Conditions that were optimised were type of DharmaFECT transfection reagent (DharmaFECT 1, 2, 3 or 4; Dharmacon), concentration of transfection reagent and concentration of siRNA molecule. Further optimisation of the transfection protocol was required in the upscaling of the process to facilitate a high throughput siRNA screen. Liquid handling robots were assessed and optimised for each step of the protocol.

As the final siRNA screen will be high throughput, a suitable output assay needs to be selected that is relevant to the overall study as well as being suitable for a high throughput process. The primary aim of this study is to develop a functional siRNA screen to identify novel therapeutic targets for the treatment of oesophageal malignancies. Gene expression microarray studies described in the previous chapter have identified a number of cellular processes that are associated with development of oesophageal malignancies that are also responsive to bile acids, known contributors to oesophageal carcinogenesis. The development of a final assay must be based on previously acquired knowledge relevant to the overall aim of the study and also on the availability of a robust assay suitable for a high throughput system.

4.2 Objectives

This chapter aims to further investigate cellular processes that were identified in the previous chapter as linked to DCA responsive genes involved in oesophageal adenocarcinogenesis. This aim was achieved by:

- the development of a semi-automated siRNA screening protocol to improve specificity of inhibition and to further detail and expand on the number of signalling pathways that are targeted.

- assessing the cell proliferation of cell lines representing the different stages of the oesophageal carcinogenic sequence in response to DCA exposure.
- identifying modulators of cell viability responses to DCA exposure using a small panel of chemical inhibitors.
- identifying modulators of cell proliferation in oesophageal cell lines in the absence of external stimuli.

4.3 Results

4.3.1 Development of a semi-automated siRNA transfection protocol

4.3.1.1 Assessment of accuracy and consistency of liquid handling robots

Liquid handling robots are designed to facilitate the efficient expansion of routine laboratory protocols to a high throughput level. The accuracy and consistency of all liquid handling robots must be assessed to maintain the integrity of the experimental procedures. Liquid handling robots to be tested to develop a semi-automated siRNA transfection protocol were the Matrix Hydra II™ and the Matrix WellMate™. The experimental steps involved in siRNA transfection are described in Figure 4.1 with proposed contribution of each liquid handling robot to development of a semi-automated siRNA transfection protocol.

The Matrix Hydra II™ contains 96 individual needles which dispense liquid using a positive displacement syringe based mechanism. Two sources of error due to pipetting variation can arise from this system. The first source is between individual needles on the plate with the second source being between multiple dispenses within a single needle. Both of these potential sources of error, along with the accuracy and consistency of the system were assessed using a 0.1µg/ml solution of fluorescein. Fluorescein is a well-documented, cheap and effective method used to test the accuracy of liquid handling robots

(David and Mitchell, 2006). White walled 96 well plates were used to prevent inter well interference during plate reading. The volume that was dispensed was 3µl to give a final siRNA concentration of 100nM. This dispensing step was repeated ten times. The volume chosen to be tested is the volume of siRNA that will be dispensed from the master plate into each daughter plate and is an extremely important step in the protocol.

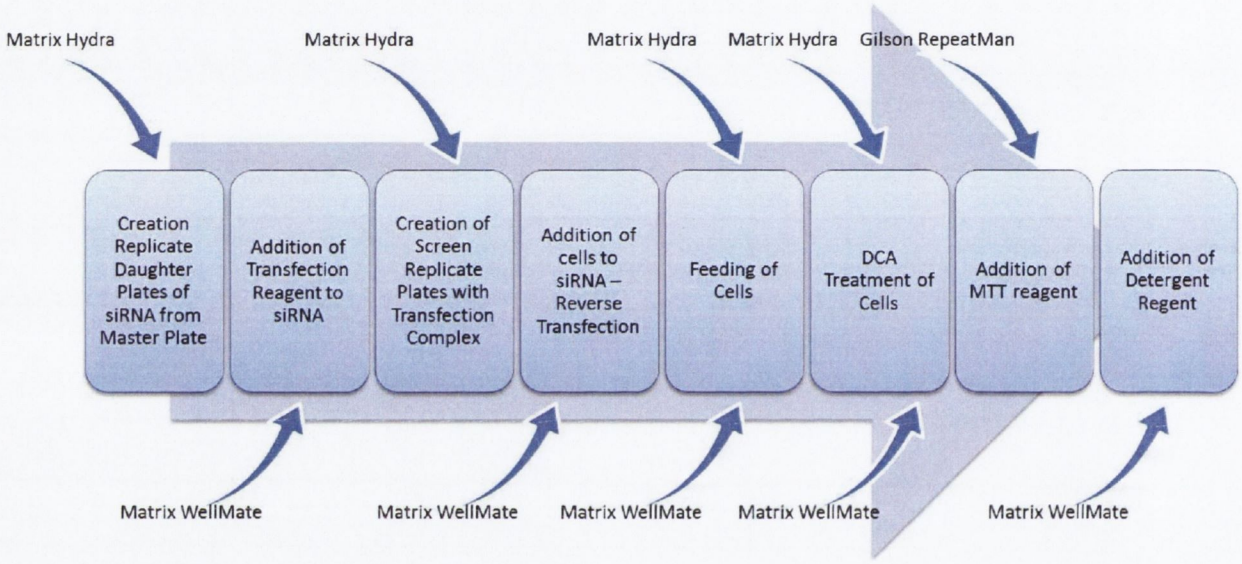


Figure 4.1: Proposed semi-automated siRNA transfection protocol using liquid handling robots. The expansion of siRNA transfection to a high throughput siRNA screening level requires adaption of each step of the protocol. It is proposed that each step in the siRNA protocol can be executed using the Matrix Hydra II™ and the Matrix WellMate™ as illustrated above. Steps that utilise both robots e.g. feeding of cells are completed using the Matrix Hydra II™ to remove media that is replaced using the Matrix WellMate™.

An accurate estimate of the volume dispensed by the Matrix Hydra II™ will be obtained by comparing the relative fluorescent units (RFU) of the dispensed volume to a standard curve of RFUs of known volumes. The standard curve will be obtained using a Gilson Repeatman™ electronic pipette. The Repeatman™ also uses a positive displacement syringe based dispense mechanism and is a gold standard in consistency and accuracy.

The mean RFUs from the ten dispensing steps for each of the 96 needles were plotted on a surface graph depicting the layout of the 96 well plate (Figure 4.2A). This graph clearly

illustrates that there are no inconsistencies between the needles. An inaccurate needle would appear on this surface graph as a peak pointing up or down. Instead, a relatively flat surface was obtained indicating consistent dispensing across the 96 needles. The range of the RFU values obtained from the 96 needles equates to a dispensed volume range of 2.96µl to 3.15µl with a mean percentage coefficient of variation (%CV) of 3.8% between the values of each well on the plate. This is within the acceptable range as suggested in the Matrix Hydra II™ manual.

The %CV for each individual needle over the ten dispensing steps was also determined and plotted on a surface graph (Figure 4.2B). A slight edge effect was noticed in row H of the plate where a small lack of consistency was observed (red) in some of the needles. These needles showed a maximum %CV of 6.6%. These needles were inspected and found to have no visible defects that would affect inconsistency. Currently, Dharmacon siRNA transfection protocols using DharmaFECT transfection reagent recommend using a final concentration of siRNA per well of 25nM. The final concentration of siRNA in each well, as utilised in previous experiments, was 100nM. This concentration of siRNA is above a saturating level which would render slight inconsistencies in dispensing as irrelevant. Taking this into consideration this level of % CV was deemed acceptable for this protocol.

The accuracy of the Matrix Hydra II™ and the Matrix WellMate™ over a broad range of volumes was also investigated. A range of volumes from 0 to 100µl was dispensed using fluorescein (0.1µg/ml) to determine an estimate of the volume dispensed. For the Hydra II™, each volume was dispensed three times and the mean value for the plate was used to plot a standard curve of RFU against volume dispensed. In the case of the Matrix WellMate™, a 96 well plate was completely filled with each volume on the standard curve. The mean RFU value was calculated. This process was repeated 3 times and a

standard curve was obtained by plotting RFU against volume dispensed. Both of these standard curves were compared to a standard curve obtained using the Gilson Repeatman pipette (Figure 4.3A). Both curves correlate well with the Pearson r value and r^2 both being greater than 0.99. A similar approach was taken using the Matrix WellMate™ to assess the accuracy of this robot (Figure 4.3B). Again, the standard curves correlated well with a Pearson r value and r^2 greater than 0.99 indicating correlation between the two standard curves.

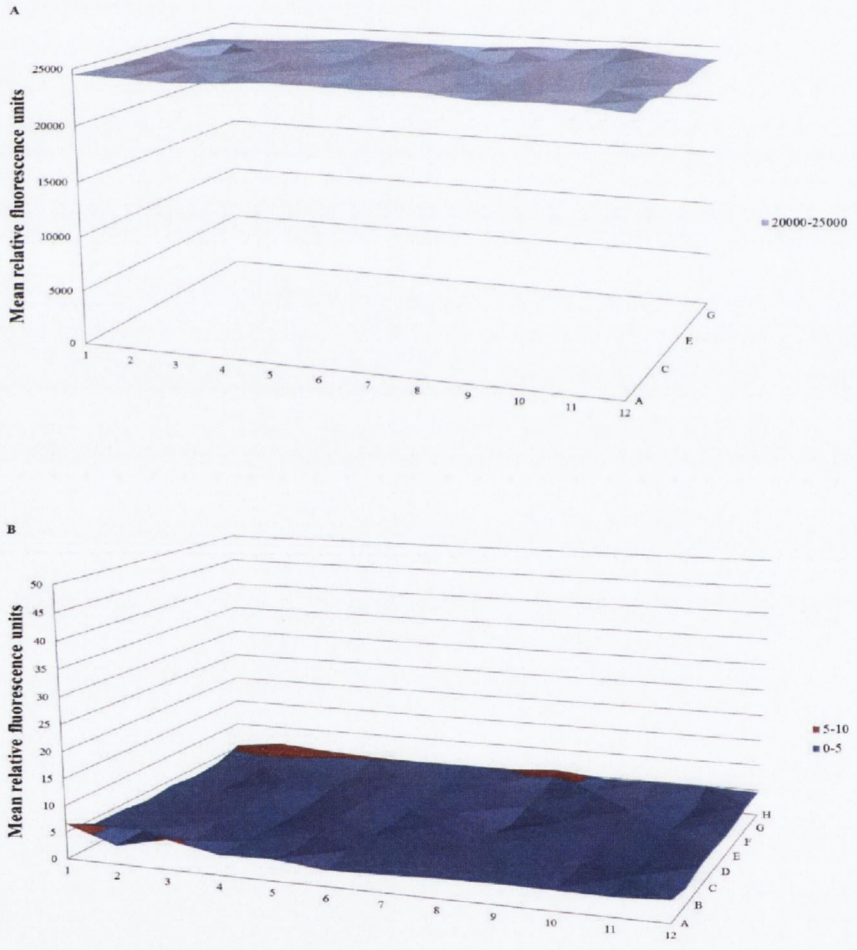


Figure 4.2: Assessment of accuracy and consistency of the Matrix Hydra II™ liquid handling robot. Fluorescein solution (0.1µg/ml) was used to assess the accuracy and consistency of the liquid handling robots. The variation between needles on the plate and the variation within individual needles as sources of error were assessed. (A) There was no significant variation observed between the 96 needles. A relatively flat surface graph was obtained, indicating consistency of dispensing between the needles. (B) A surface graph was constructed illustrating the % CV between the ten dispensing steps. This graph indicates a possible edge effect with a slightly higher level % CV observed in row H of the plate which should be neutralised by the use of an excess of siRNA in each well.

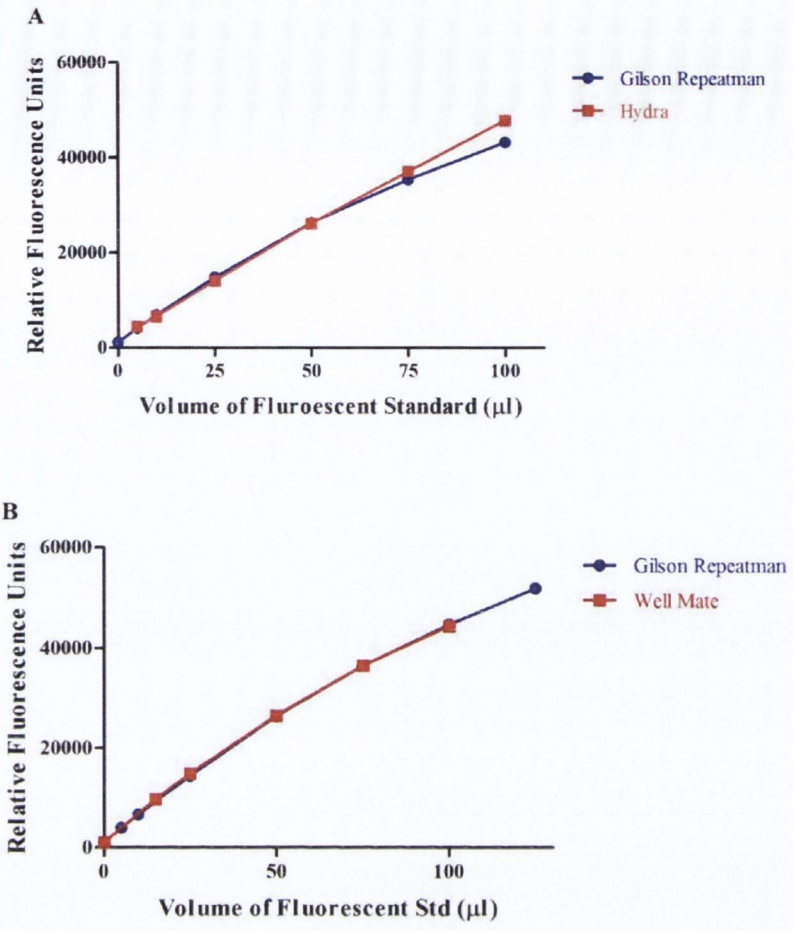


Figure 4.3: Accuracy of Matrix Hydra II™ and Matrix WellMate™ Liquid Handling robots. The accuracy of the Matrix Hydra II™ and the Matrix WellMate™ was determined using a standard curve of RFUs plotted against volume of fluorescein dispensed. The curves obtained were plotted with a standard curve obtained using a Gilson Repeatman pipette. Pearson correlation values for (A) the Hydra II™ compared with the Repeatman and (B) the WellMate™ compared with the Repeatman were both greater than 0.99, indicating a strong correlation. Both r^2 values were also >0.99.

The Matrix WellMate™ operates using a peristaltic pump mechanism which dispenses liquid through eight plastics tubes that are positioned over a moving stage which holds a 96 well plate. The stage moves horizontally to allow multiple dispensing steps from each of the tubes to complete an entire 96 well plates. The accuracy and consistence of each tube of the Matrix WellMate™ was tested using a gravimetric method. Each WellMate™ tube was used to dispense ten volumes of 50µl of water into one microcentrifuge tube giving an estimated total volume in the microcentrifuge tube of 500µl. This was repeated three times

for each tube of WellMate™. The mass of the water in the microcentrifuge tube was used to estimate the volume dispensed by each tube of the WellMate™ (Figure 4.4).

The maximum estimated volume dispensed was from tube E which had an estimated total volume of 528µl. This indicates that this tube had dispensed an extra 27µl approximately after ten dispensing steps which correspond to an average of an extra 2.7µl per 50µl dispensed into each well. This range of variability was deemed acceptable in the context of this protocol.

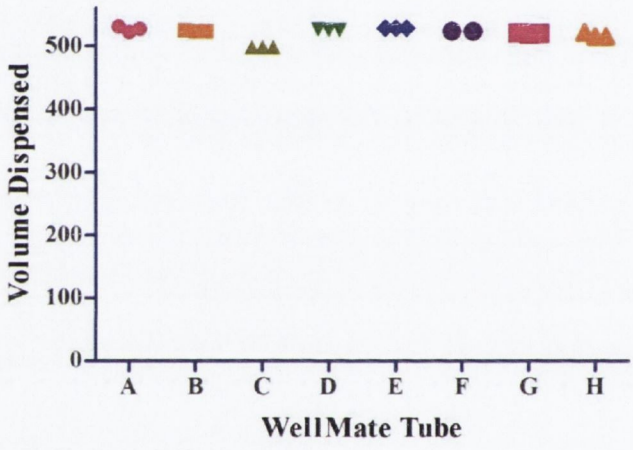


Figure 4.4: Accuracy of the Matrix WellMate™ assessed using gravimetric method. Each tube of the Matrix WellMate™ was used to dispense ten volumes of 50µl of water into one microcentrifuge tube (repeated three times). An estimate of the volume dispensed was calculated from the mass of the water dispensed. The volumes dispensed range from 498µl (Tube C) to 528µl (Tube E). This indicates that the maximum difference between a single dispensing step from any two tubes will be at most 3µl.

4.3.1.2 Delivery method of DCA solution using liquid handling robots

Routine exposure of cells in culture to DCA involves replacement of all complete medium with experimental medium containing the relevant concentration of DCA. In high throughput protocols, the mechanisms employed by liquid handling robots do not facilitate the removal of all medium from tissue culture plates. In order to overcome this issue, an alternate method of DCA exposure was tested. Typically in isolated experiments, 100µl of a solution of DCA at the final concentration required is added to a 96 well plate of cultured

cells. This would hamper high throughput addition of solution. Therefore, the removal of 50% of culture media followed by addition of a 2X solution of DCA of the require concentration was tested. The alternate delivery method for DCA did not affect the cell viability when compared to the previously used method (Figure 4.5).

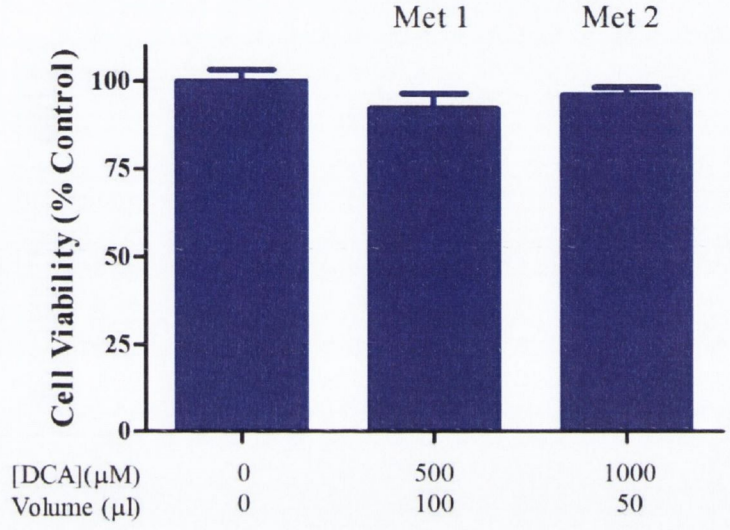


Figure 4.5: Cell viability of GOhTRT cells using two delivery methods for DCA. The delivery method used to exposure GOhTRT cells to DCA does not affect cell viability to a significant level. DCA was added to GOhTRT cells using two methods:

1. 100μl media was removed - 100μl 500μM DCA was added.
2. 50μl media was removed - 50μl of 1 000μM DCA was added.

Both methods give a final volume of 100μl of 500μM.

4.3.2 High throughput siRNA library screen assay development 1 - Identification of modulators of DCA signalling in oesophageal cell lines

4.3.2.1 Cell viability of oesophageal cell lines following DCA treatment

DCA responsive genes with altered gene expression patterns during oesophageal adenocarcinoma development have been identified using microarray technology (Figure 3.10). A systems biology approach was utilised to determine the functional relevance of this cohort of genes. Ingenuity Pathways Analysis (IPA) software identified biological processes that were associated with this cohort. Among the top ranked biological

processes that were identified were cell death and cell growth & proliferation (Figure 3.16). Variations in the cell proliferation of oesophageal cell lines in response to DCA were assessed using an MTT Cell Proliferation assay (ATCC, Middlesex, UK). This protocol required a minimal number of pipetting steps which reduces potential sources of experimental variation. The simplicity of the protocol makes this kit an ideal assay for use in high throughput experiments. The cell lines used in this experiment represented *in vitro* each stage of the oesophageal carcinogenic sequence from normal squamous (Het-1A cell line) through metaplasia (QhTRT cell line) to dysplasia (GOhTRT cell line) and adenocarcinoma (SKGT4 cell line). These cell lines were exposed to a range of concentrations of DCA (0μM to 1000μM) using a series of end points.

DCA exposure reduced the cell viability of each cell line in a dose dependant fashion (Figure 4.6, Table 4.1). The viability of Het-1A, QhTRT, GOhTRT and SKGT4 cells following exposure to 500μM DCA was approximately 70%, 30%, 95% and 110% respectively. This represents a statistically significant reduction in cell viability of the Het-1A and QhTRT cells but not in the GOhTRT and SKGT4 cells compared to untreated resting controls (p-value <0.05, Student's t-test). These data indicate that the cell lines representing the early stages of the carcinogenic sequence (Het-1A and QhTRT cells) are more sensitive to DCA. Resistance of GOhTRT cells to DCA was further confirmed using an extended time course of 8 hours. An examination of cell proliferation of GOhTRT cells following exposure to DCA ([500μM]) for 8 hours demonstrated a slight decrease in cell viability to ~92% (p-value < 0.05) (Figure 4.7). The viability of the GOhTRT cells after 4 and 6 hours exposure to DCA ([500μM]) was ~100% and ~107% respectively. This experiment was repeated 4 times with 3 replicate wells in each experiment demonstrating reproducibility.

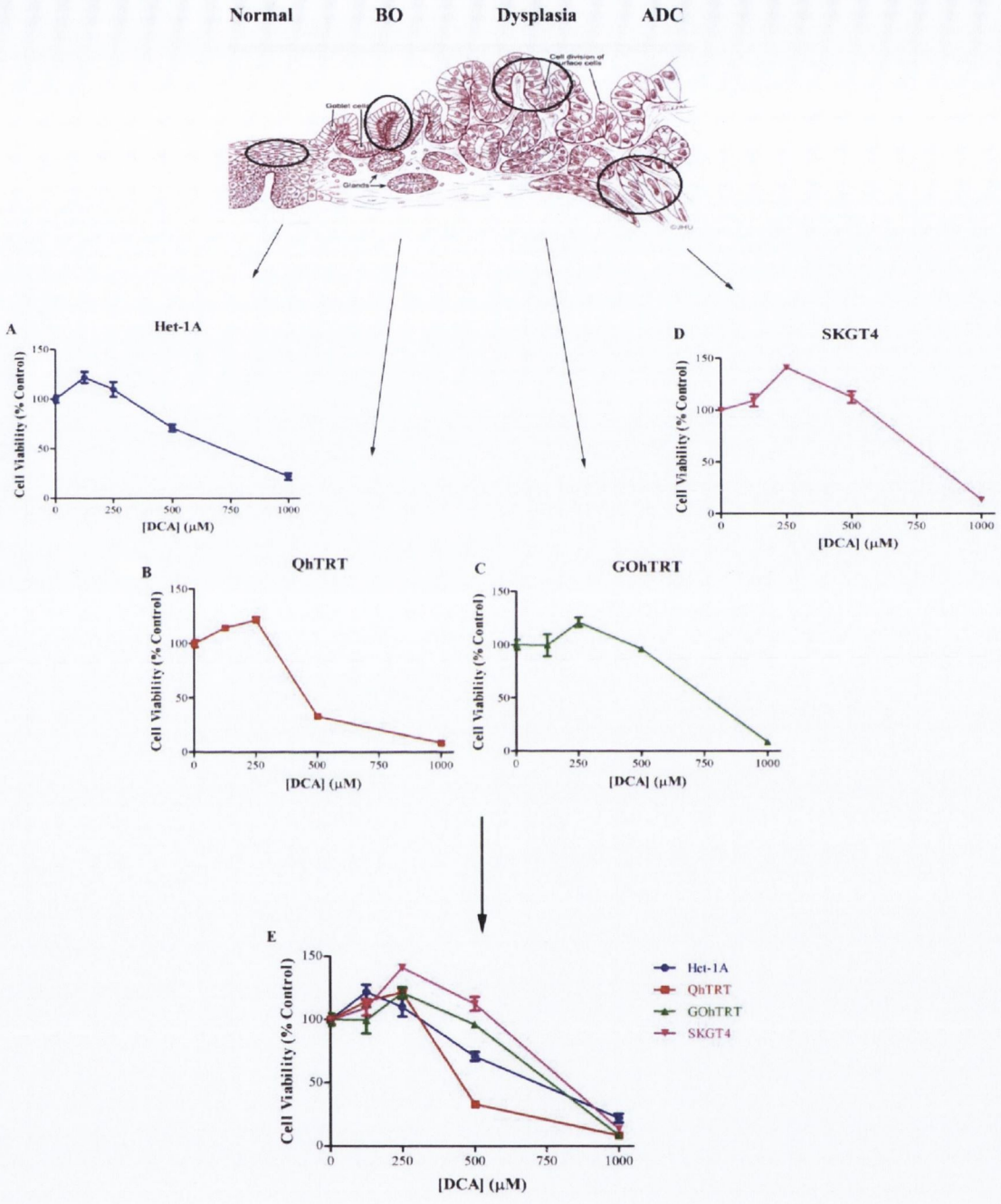


Figure 4.6: Dose responsive curve of oesophageal cell lines exposed to DCA for 6 hours. Oesophageal cell lines were exposed to DCA for 6 hours over a range of concentrations (0μm - 1000μM). Cell viability was assessed using an MTT assay. Cell lines representing the early stages of the carcinogenic sequence, (A) Het-1A and (B) QhTRT, were most sensitive to DCA ([500μM]) as cell viability was significantly reduced to 70% and 30% respectively when compared to resting controls. The cell viability of (C) GOhTRT and (D) SKGT4 cell lines was not reduced at this concentration of DCA. (E) A composite graph illustrating viability of all cell lines following DCA treatment demonstrated that the viability of Het-1A and QhTRT cells was significantly lower than that of the GOhTRT and SKGT4 cells (p-value < 0.05, Student's t-test; Table 4.1). (*Modified from Koppert et al 2005.)*

Table 4.1: Statistical significance (p-value) of alterations in cell viability resulting from exposure of each cell line to DCA (6 hours, [500μM])

	Het-1A	QhTRT	GOhTRT	SKGT4	DCA [500μM] compared to cell line matched untreated control
Het-1A	1	-	-	-	0.010646
QhTRT	0.002299	1	-	-	7.36E-05
GOhTRT	0.019772	0.000249	1	-	0.589718
SKGT4	0.01176	0.00018265	0.105434	1	0.093846

p-values determined using Student's t-test; compared to dose match [500μM] in other cells lines or to cell line matched untreated controls, red indicates p-value < 0.05

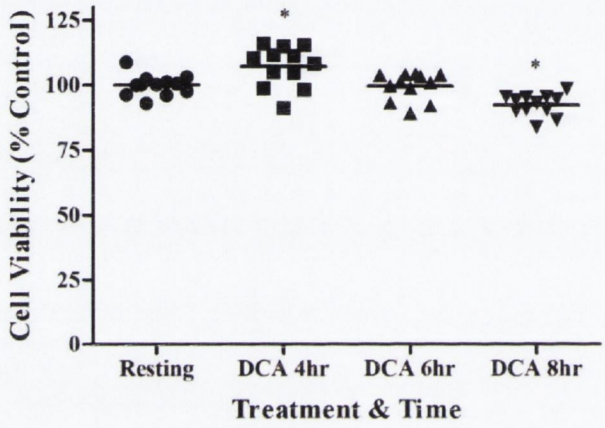


Figure 4.7: Cell viability of GOhTRT cells following DCA exposure [500μM] for 4, 6 or 8 hours. GOhTRT cells were exposed to DCA [500μM] for 4, 6 or 8 hours. Cell viability was assessed using an MTT assay. Cell viability of GOhTRT cells was significantly increased to ~110% following 4 hours exposure to DCA ([500μM]). Cell viability returned to a level similar to untreated resting cells after 6 hours and a slight decrease in cell viability to ~90% of resting control was observed after 8 hours. This experiment was repeated 4 times with 3 replicate wells in each experiment (* = p-value < 0.05 determined using Student's t-test.)

4.3.2.2 Identification of modulators of resistance to DCA induced reduction in cell viability using a panel of chemical inhibitors.

GOhTRT cells have displayed resistance to a DCA induced reduction in cell viability (Figure 4.6, Figure 4.7). A panel of chemical inhibitors were selected to sensitise these resistant cells to a DCA mediated reduction in cell viability and as such identify modulators of this resistance. Het-1A and GOhTRT cells were pre-treated with each inhibitor (30 mins) followed by a co-treatment with the inhibitor and DCA (6 hours, 500μM). Cell viability was assessed using an MTT cell viability assay. DCA treatment at

a concentration of 1000μM was used as a positive control. Each treatment was carried out in triplicate. Statistical significance was determined using the Student's t-test and p-values <0.05 were deemed significant. The results of this chemical inhibitor screen are summarised in Table 4.2.

B-cell lymphoma-2 (BCL2) like-1 (BCL2L1), also known as BCL-XL is an anti-apoptotic protein which has been demonstrated to have increased expression levels as oesophageal adenocarcinoma develops (van der Woude *et al.*, 2002). BCL-XL was inhibited using the chemical inhibitor BH3I-1. The viability of Het-1A cells was largely unaffected by inhibition of BCL-XL in conjunction with DCA (Figure 4.8 A). In contrast, inhibition of BCL-XL in GOhTRT cells resulted in a dose dependent decrease in cell viability following co-treatment with DCA (Figure 4.8 B). This would indicate that BCL-XL is involved in conferring resistance to DCA cell death in GOhTRT cells. Another anti-apoptotic protein, BCL2 was inhibited using HA14-1 chemical inhibitor. Both Het-1A and GOhTRT cells exhibited a decrease in cell viability following co-treatment with HA14-1 and DCA at higher concentrations of the inhibitor (> 25μM & 100μM respectively; Figure 4.8 C&D).

Jak family tyrosine kinases have key roles in the regulation of cell proliferation, differentiation, migration and survival and could be involved in conferring cell death resistance in GOhTRT cells. Het-1A cells did not display altered levels of cell viability following Jak family inhibition by AG490 with or without co-treatment with DCA (Figure 4.8E). Jak inhibition did result in a slight increase (~20%) in the cell viability of GOhTRT cells at an inhibitor concentration of 100μM (Figure 4.8F). Phosphatidylinositol 3-kinases (PI3-K) play a pivotal role in the regulation of motility, proliferation and survival. The PI3-K inhibitor Wortmannin did not result in a significant alteration in either Het-1A or GOhTRT cell viability when co-treated with DCA (Figure 4.8G&H). Viability of GOhTRT cells was decreased by approximately 20% following treatment with the

inhibitor alone. Protein kinase C (PKC) has again been implicated as a key modulator of cell proliferation and differentiation and is another candidate for mediating DCA signalling in oesophageal cells. PKCs have also been demonstrated to be downregulated in colon adenocarcinomas and in early stages of intestinal carcinogenesis. Inhibition of PKC in Het-1A and GOhTRT cells using Bisindolylmaleimide I (Bis I) with and without a DCA co-treatment did not alter cell viability significantly from cells that were untreated or treated with DCA alone (Figure 4.8I&J).

The NF-κB transcription factor has a role in inflammation, autoimmune response, cell proliferation and apoptosis. Many cancer cells exhibit constitutively active NF-κB which protects cell from apoptosis and may enhance growth activity. Functionally active NF-κB appears to be a requirement in the survival of Het-1A cells. At concentrations of the NF-κB inhibitor, Bay 11-7085, greater than 10μM, these cells have reduced viability similar to that observed in cells treated with the positive control (10-15%). This occurs in cells with and without a DCA co-treatment (Figure 4.8K). Viability of GOhTRT cells in not altered following NF-κB inhibition (Figure 4.8L).

Mitogen-Activated Protein (MAP) kinases mediate signal transduction from the cell surface to the nucleus and regulate important cellular processes such as inflammation, apoptosis, oncogenic transformation, tumour cell invasion and metastasis. MAP kinase kinases (MEK) are involved in the MAP kinase signalling cascade. The p38 MAP kinase is activated in response to inflammation. Viability of Het-1A cells was unaltered following inhibition of MEK using both PD98059 and U0126 with and without a DCA co-treatment (Figure 4.8M&O). GOhTRT cell viability was unaltered following treatment with PD98059 alone but a co-treatment of PD98059 and DCA caused an approximately 20% initial decrease in cell viability at low concentration of PD98059 (Figure 4.8 N).

Increasing concentration of PD98059 resulted in an increase in the cell viability of GOhTRT cells. This initial decrease in GOhTRT cell viability was not repeated using the U0126 MEK inhibitor. Cell viability was increased by between 10 and 20% approximately with and without a DCA co-treatment with U0126 (Figure 4.8P). There is no significant alteration in cell viability of Het-1A cells in response to p38 inhibition with or without DCA co-treatment (Figure 4.8Q). Inhibition of p38 in GOhTRT cells using SB203580 resulted in a slight reduction of cell viability (~25%). Co-treatment with SB203580 and DCA had mixed response in GOhTRT cells but cell viability did not deviate from cells treated with DCA alone by more than ~10% (Figure 4.8R).

This chemical inhibitor screen demonstrated that inhibition of the anti-apoptotic protein BCL-XL sensitised GOhTRT cells to a DCA mediated reduction in cell viability most significantly. Inhibition of BCL2, another anti-apoptotic protein, also sensitised GOhTRT cells but to a lesser extent. This series of experiments defined that BCL-XL and BCL2 are capable of mediating resistance to DCA induced cell death in GOhTRT cells.

Table 4.2: Summary of cell viability of Het-1A and GOhTRT cells following cotreatment with a panel of chemical inhibitors and DCA (6 hours; [500μM])

Target	Inhibitor	Effect on Het-1A Cell Viability following DCA exposure	Effect on GOhTRT Cell Viability following DCA exposure
BCL-XL	BH3I-1	Unchanged	Decreased
BCL2	HA14-1	Decreased	Decreased (at high inhib conc)
Jak Family	AG490	Unchanged	Increased (at high inhib conc)
PI3K	Wortmannin	Unchanged	Unchanged
NF-κB	Bay 11-7085	Decreased	Unchanged
PKC	Bisindolylmaleimide I	Unchanged	Unchanged
MEK	PD98059	Unchanged	Decrease low inhib conc Increase high inhib conc
MEK	U0126	Unchanged	Unchanged
p38	SB203580	Unchanged	Unchanged

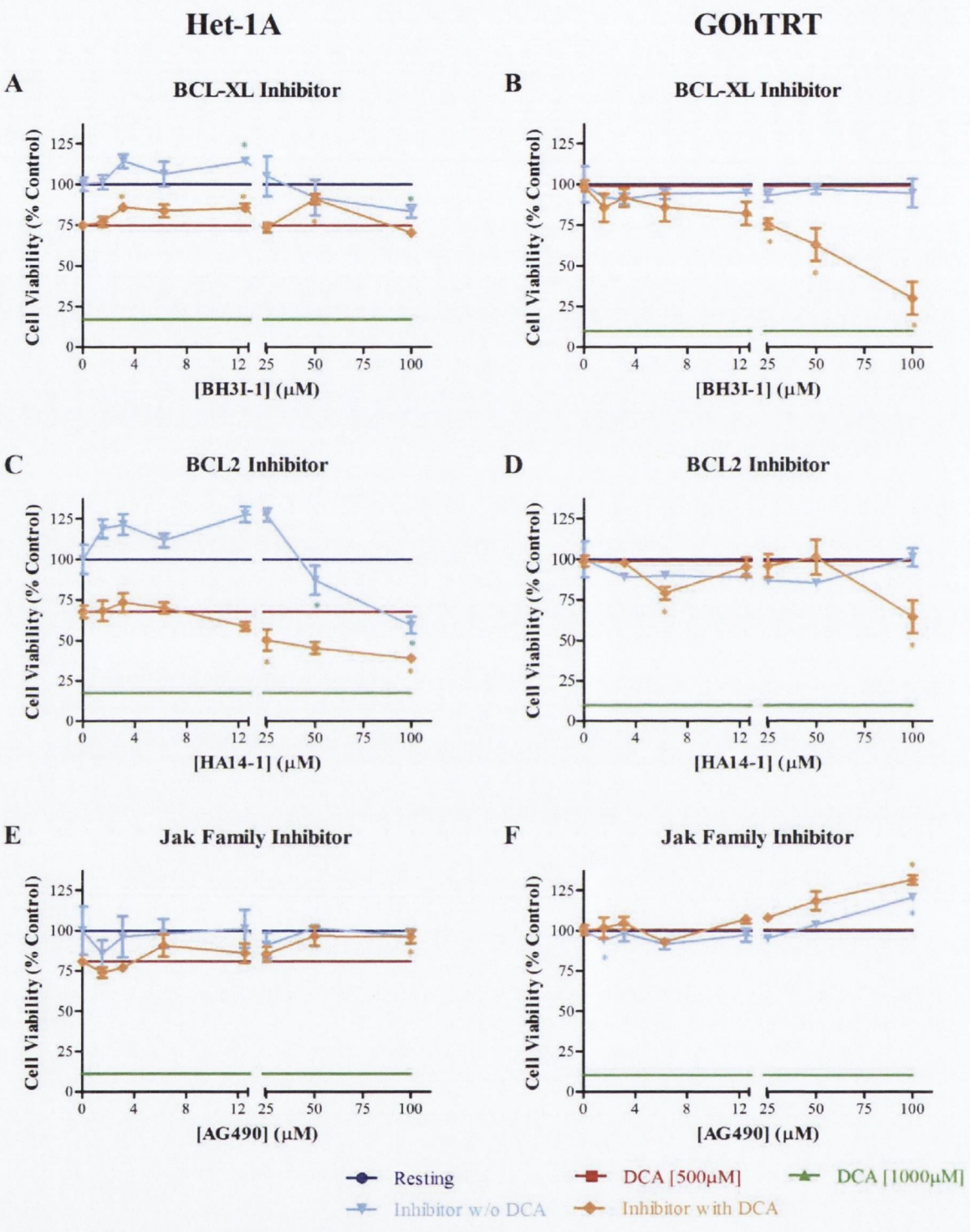


Figure 4.8 continued overleaf

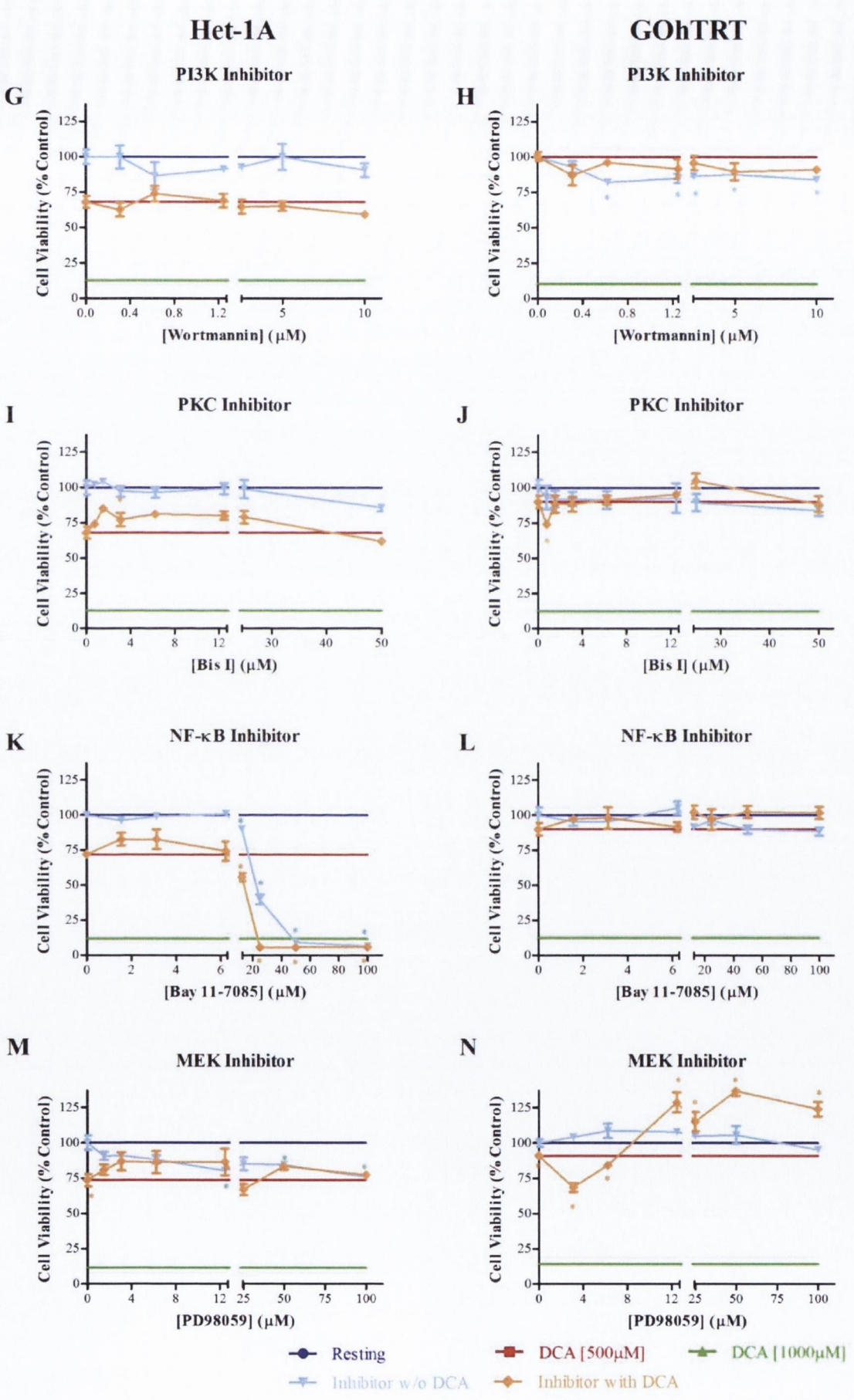


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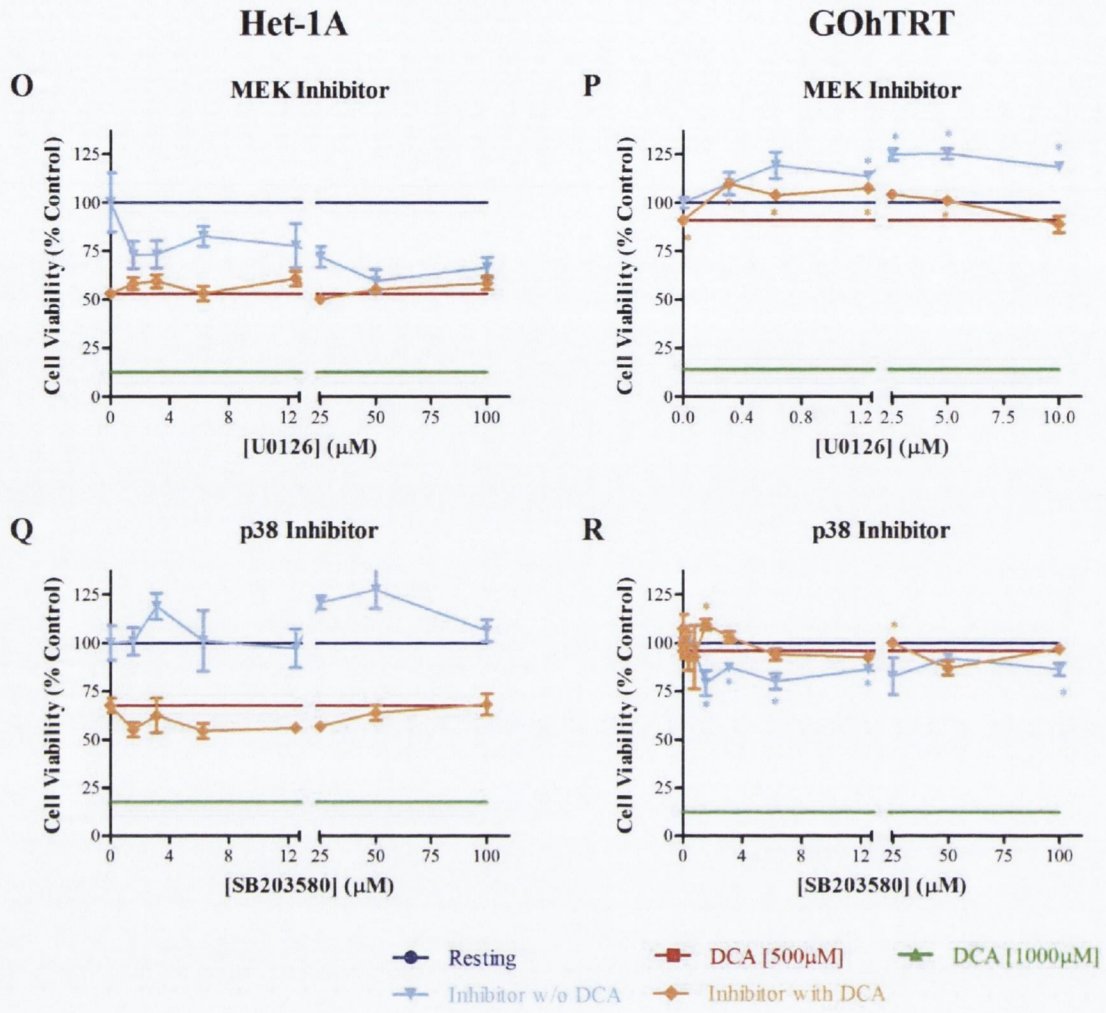


Figure 4.8: A small chemical inhibitor screen identifies BCL-XL as a modulator of resistance to DCA induced cell death in GOhTRT dysplastic oesophageal cells. Cell lines were pre-treated (30 mins) with each of the chemical inhibitors, followed by cotreatment with the inhibitor and DCA (Table 4.2). Cell viability was assessed using an MTT assay. DCA ([1000μM]) was used as a positive control. The cell viability of (B) GOhTRT cells was significantly reduced in a dose dependent fashion following co-treatment with DCA and the BCL-XL inhibitor. Inhibition of BCL2 in (C) Het-1A and (D) GOhTRT cells further sensitised both cell lines to DCA. Inhibition of Jak family resulted in a slight increase in cell viability of (F) GOhTRT cells at the highest concentration (100μM) of the inhibitor. Cell viability (H) GOhTRT cells was slightly decreased (~80%) following inhibition of PI3K. (K) Het-1A cell viability was reduced to a level that was comparable to the positive control following inhibition of NF-κB (<15%). Cell viability of (N) GOhTRT cells following co-treatment with the MEK inhibitor PD98059 and DCA was reduced at low concentrations and increased at high concentrations of the inhibitor. Inhibition of MEK using U0126 in the (P) GOhTRT cells resulted in a slight increase of cell viability (~120%) with and without co-treatment with DCA. Het-1A cell viability was unaffected by inhibition of (A) BCL-XL, (E) Jak Family proteins, (G) PI3K, (I) PKC, (M, O) MEK and (Q) p38. GOhTRT cell viability was unaffected by inhibition of (J) PKC, (L) NF-κB and (R) p38 (* p-value < 0.05, Student's t-test.).

4.3.2.3 Development of a positive control for a semi-automated siRNA screen investigating modulators of resistance to DCA induced cell death

GOhTRT cells exhibit resistance to DCA mediated cell death which can be overcome using chemical inhibitors to BCL-XL or BCL2. The effect of specific inhibition of BCL-XL, BCL2 and MCL1 was carried out using siRNAs targeting the mRNA of each of these anti-apoptotic genes. Cell viability of GOhTRTs were assessed using an MTT assay 72 hours after transfection of the siRNA and following exposure of GOhTRTs to DCA for 6, 8 or 10 hours (Figure 4.9). GOhTRT cells were sensitised to DCA induced cell death following BCL-XL inhibition in a time dependant manner. Inhibition of BCL2 (Figure 4.9A) and MCL1 (Figure 4.9B) did not result in a significant sensitisation of GOhTRT cells to DCA induced cell death. Inhibition of BCL-XL may now be utilised as a positive control in a semi-automated siRNA library screen.

4.3.3 High throughput siRNA library screen assay development 2 - Identification of modulators of oesophageal cell line proliferation in the absence of external stimuli

Preliminary investigations were carried out to develop an siRNA library screen to examine proliferative potential of a dysplastic oesophageal cell line in the absence of external stimuli. A panel of siRNA targets were established in a search for a positive control to be incorporated into this siRNA library screen. These targets were selected based on findings from the microarray gene expression analysis described in the previous chapter, a literature search to select important candidates in development of OAC and molecules involved in processes associated with carcinogenesis. Individual gene targets such as growth differentiation factor 15 (GDF15) and GATA binding protein 6 (GATA6) were selected from the previously described GEM study. Cell processes such as cell death and cell proliferation, associated with the hallmarks of cancers and identified in the GEM study,

were also selected for inclusion in the panel. Representative of these processes were the anti-apoptotic genes BCL-XL and MCL1.

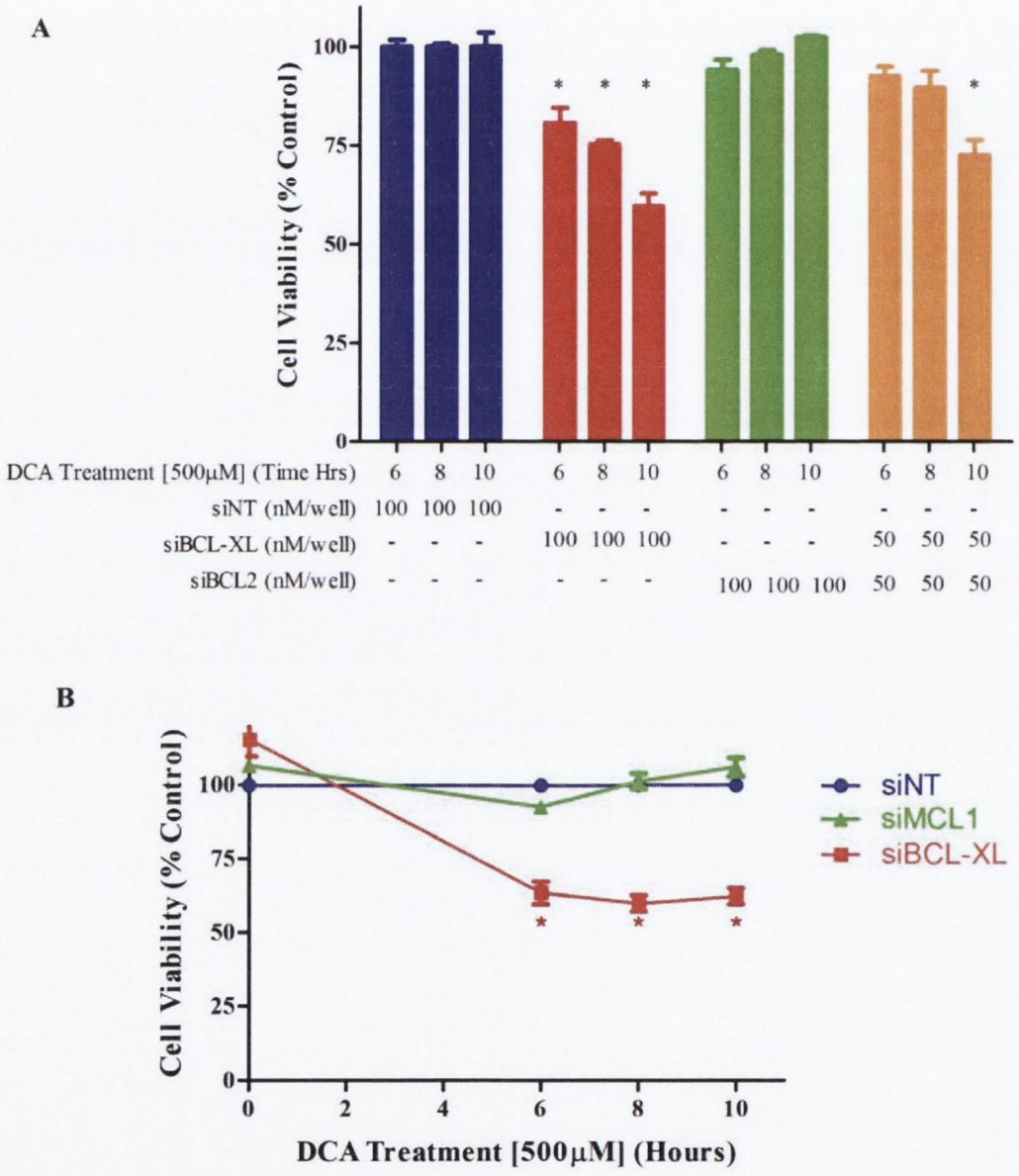


Figure 4.9: Viability of GOhTRT cells following targeted inhibition of BCL-XL, BCL2 and MCL1 in conjunction with exposure to DCA. Cell viability was determined using an MTT assay following 72 of targeted inhibition using siRNA and 6, 8 and 10 DCA exposure. Specific inhibition of (A) BCL-XL by siRNA in GOhTRTs resulted in sensitisation of the cells to DCA induced cell death in a time dependent manner. Specific siRNA inhibition of (A) BCL2 and (B) MCL2 did not result in any sensitisation of GOhTRT cells to DCA induced cell death. (* p-value < 0.05, Student's t-test; siNT: non-targeting control siRNA).

The remainder of the panel of siRNA targets were selected based on a literature search to identify genes involved in OAC development. Potential regulators of the CDX2, an important transcription factor in BO development and possibly tumour suppression, were identified by this literature search. These molecules include HNF4α, HIF-1α and GATA6. This panel of siRNA targets was examined to identify potential positive controls that could be incorporated into an siRNA library screen to identify a panel of potential therapeutic targets to inhibit cell proliferation (Table 4.3).

Table 4.3: Genes associated with progression through M-D-A sequence.

Gene Name	Gene Symbol	Source
Adenomatous Polyposis Coli	APC	(Clément *et al.*, 2007)
Cyclin D1	CCND1	(Bani-Hani *et al.*, 2000)
Caudal type homeobox transcription factor 1	CDX1	(Wong and Fitzgerald, 2005)
Caudal type homeobox transcription factor 2	CDX2	(Huo *et al.*, 2010)
v-myc myelocytomatosis viral oncogene homolog (avian)	c-myc	(Stairs *et al.*, 2008)
v-erb-b2 erythroblastic leukaemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog (avian)	ERBB2	(Arrington *et al.*, 2009)
GATA binding protein 6	GATA6	From Gene Expression Microarray - Chapter 3
Growth differentiation factor 15	GDF15	From Gene Expression Microarray - Chapter 3
Hypoxia-inducible factor 1, alpha subunit	HIF1α	(Griffiths *et al.*, 2007)
Hepatocyte nuclear factor 4, alpha	HNF4α	(Benahmed *et al.*, 2008)
Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p50)	NF-κB1 aka p50	(Huo *et al.*, 2010)
Prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	PTGS2 aka COX2	(Cheong *et al.*, 2004)
v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3 (p65)	RELA aka p65	(Kim *et al.*, 2002)
Tribbles homolog 3 (Drosophila)	TRIB3	From Gene Expression Microarray - Chapter 3
B-cell lymphoma 2 like 1	BCL2L1 aka BCL-XL	(van der Woude *et al.*, 2002)
Myeloid cell leukaemia sequence 1 (BCL2 related)	MCL1	(van der Woude *et al.*, 2002)

4.3.3.1 Assessment of cell proliferation of GOhTRT cells following specific inhibition by siRNA of a panel of targets

A panel of gene targets was specifically inhibited in GOhTRT cells using siRNA molecules. Of the 16 targets that were investigated, inhibition of 14 of these targets resulted in a significant (p-value < 0.05) reduction in cell viability (Figure 4.10; Table 4.4).

These targets were HNF4α, PTGS2, NF-κB1 (p50 subunit of NF-κB), RELA (p65 subunit of NF-κB), APC, CDX2, CCND1, MYC, ERBB2, GATA6, GDF15, BCL-XL, TRIB3 and MCL1. The targeted inhibition of GATA6, BCL-XL and MCL1 resulted in three of the most significant decreases in cell viability (46%, 44% and 33% reduction respectively). As these three molecules resulted in the greatest decrease in the cell viability of GOhTRT cells, further investigations were carried used using GATA6, BCL-XL and MCL1.

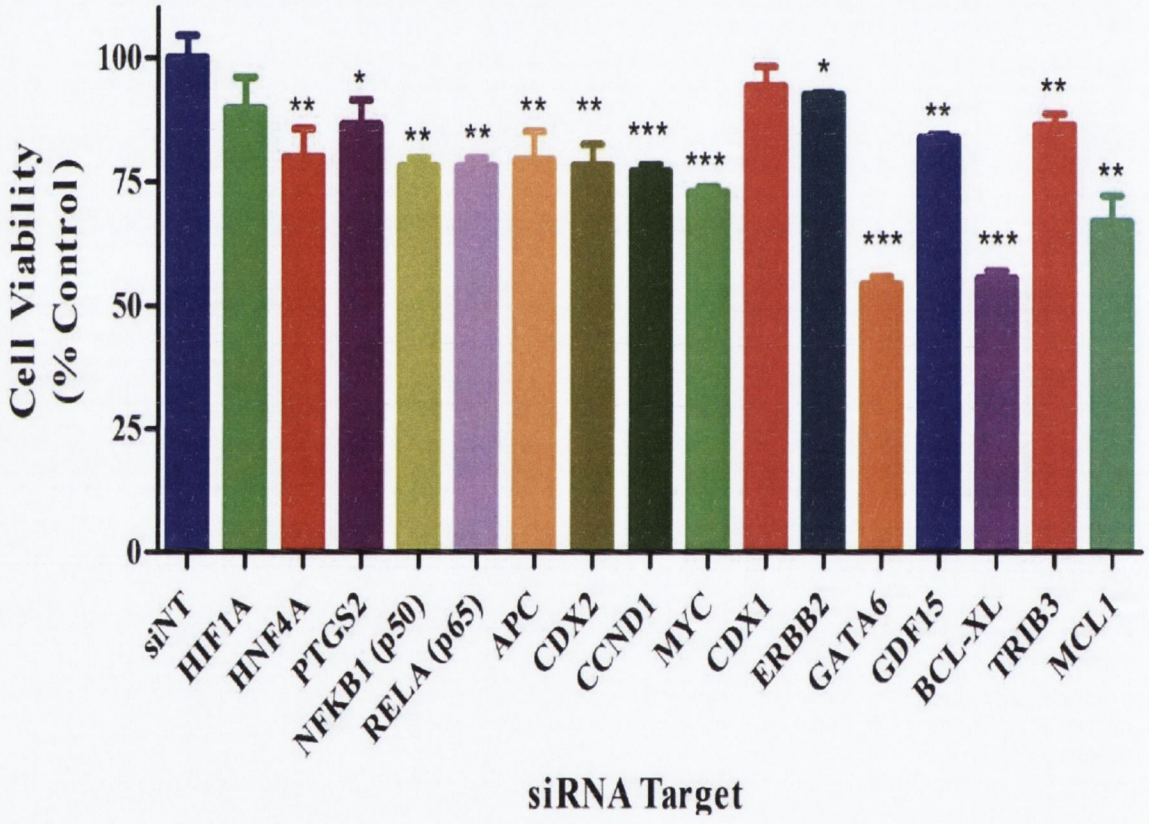


Figure 4.10: Cell viability of GOhTRT cells following targeted inhibition of a panel of genes. Cell viability of GOhTRT cells were reduced significantly following specific inhibition of HNF4α, PTGS2, NF-κB1 (p50 subunit of NF-κB), RELA (p65 subunit of NF-κB), APC, CDX2, CCND1, MYC, ERBB2, GATA6, GDF15, BCL-XL, TRIB3 and MCL1. Inhibition of GATA6, BCL-XL and MCL1 resulted in the most substantial reduction in cell viability (< 75%) (Table 4.4). Initial seeding density was 20 000 cells per well. (* p-value < 0.05; ** p-value < 0.009; *** p-value < 0.001, Student's t-test; siNT: non-targeting control siRNA.)

Table 4.4: Cell viability of GOhTRT following specific inhibition of a panel of gene targets

Target	% Cell Viability	p-value
APC	80	0.075
BCL-XL	56	<0.0001
CCND1	77	0.0010
CDX1	94	0.1634
CDX2	78	0.0035
ERBB2	93	0.0424
GATA6	54	<0.0001
GDF15	84	0.0032
HIF1α	90	0.0809
HNF4α	80	0.0084
MCL1	67	0.0011
MYC	73	0.0005
NF-κB1 (p50)	78	0.0013
PTGS2	87	0.0236
RELA (p65)	78	0.0013
TRIB3	86	0.0089

4.3.3.2 Specific inhibition of GATA6, MCL1 and BCL-XL in GOhTRT and Het-1A cells

As previously described, specific inhibition of GATA6, MCL1 and BCL-XL in GOhTRT cells resulted in a significant (p-value < 0.002) reduction in the cell viability of GOhTRT cells (Figure 4.10). This reduction in cell viability of GOhTRT cells was further confirmed in an investigation to compare the effect of inhibition of GATA6, MCL1 and BCL-XL on the proliferation of Het-1A cells (Figure 4.11A). Interestingly, specific inhibition of these three molecules did not result in a reduction in the cell viability of Het-1A cells (Figure 4.11B). These data suggest that GATA6, BCL-XL and MCL1 are involved in the cell proliferation of an oesophageal dysplastic cell line but not in normal oesophageal cells. This may be of importance as a novel therapeutic strategy targeting these molecules would result in a reduction in viability of dysplastic cells but leave normal cells unharmed. Inhibition of these three genes individually results in a significant decrease in cell viability of GOhTRT cells but does not completely eradicate these cells. Further investigations

were carried out to examine the effect of simultaneously inhibiting more than one of these targets was investigated.

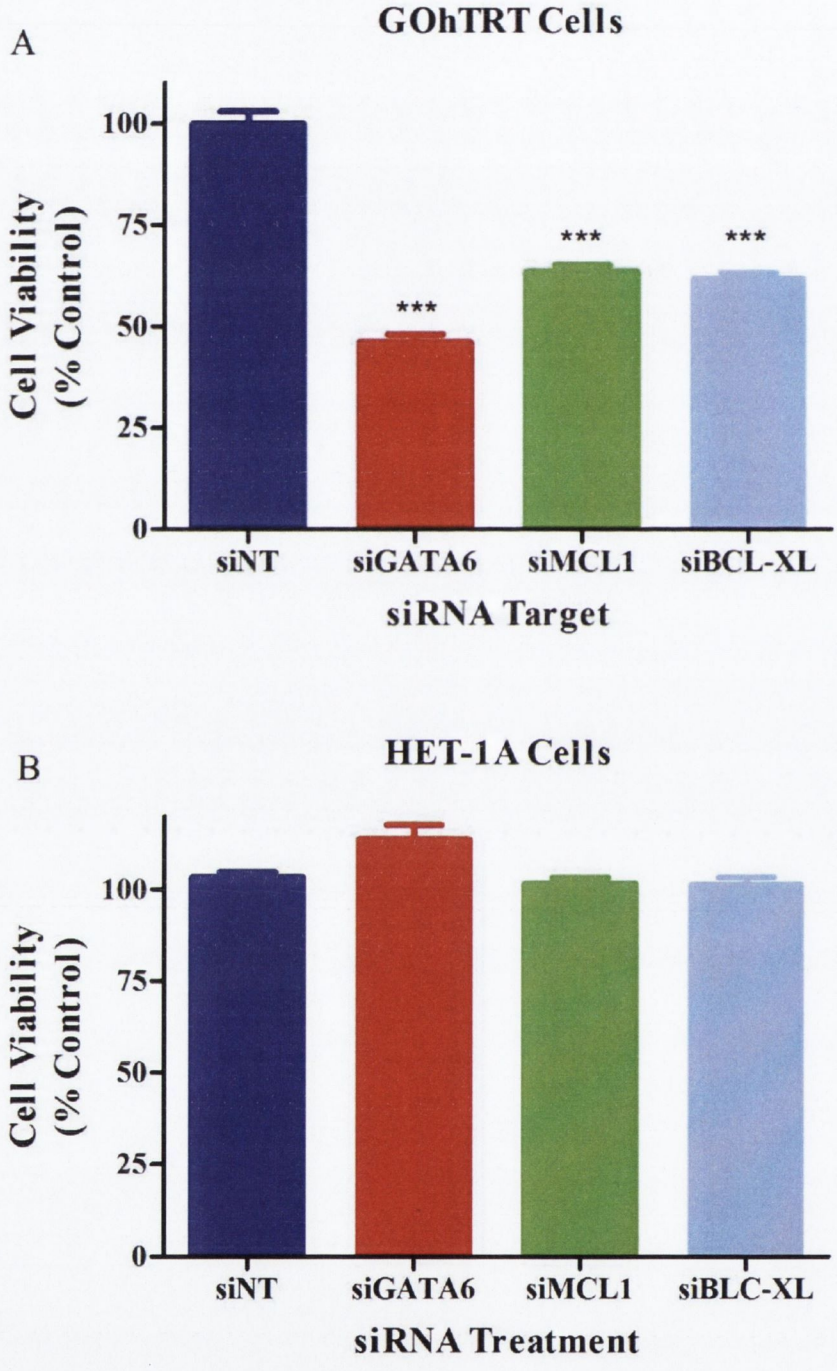


Figure 4.11: Specific inhibition of GATA6, MCL1 and BCL-XL in GOhTRT by siRNA results in a decrease in cell viability of GOhTRT cells but not Het-1A cells. (A) The cell viability of GOhTRT cells was significantly reduced following inhibition of GATA6, MCL1 and BCL-XL to 46%, 64% and 62% respectively. (B) The cell viability of Het-1A cells was not reduced following inhibition of these three molecules. The initial seeding density was 20 000 cells per well. (*** p- value < 0.0005. Students t-test; siNT: non-targeting siRNA control)

4.3.3.3 Combined inhibition of GATA6, BCL-XL and MCL1 by siRNA in GOhTRT cells

An important factor to be considered when combining different siRNA pools is to keep the final concentration of siRNA as low as possible to avoid a toxic effect on the cells or an increase in off-target effects. In order to determine whether a decrease in the concentration of each individual siRNA would result in a significant difference in resulting phenotype, the effect of reducing the concentration of the siRNA in each well from 100nM to 50nM was investigated (Figure 4.12). Cell viability following specific inhibition by each siRNA was compared at both concentrations. There was no significant difference in the reduction of cell viability resulting from siRNA inhibition at both concentrations of siRNA. These data indicate that a reduction in the final concentration of siRNA in each well does not significantly reduce efficacy. In future experiments using a combination of siRNA molecules, the starting concentration of each individual siRNA can be reduced from 100nM to 50nM while maintaining a reduction in cell viability. The concentration of non-targeting siRNA (siNT) controls was always matched with the total concentration of the siRNA in the experimental wells.

The three siRNA pools targeting GATA6, MCL1 and BCL-XL were combined using two concentrations of each individual siRNA (50 & 100nM) giving final siRNA concentrations of 150nM and 300nM respectively (Figure 4.13). Combinations of the three siRNA pools using two concentrations of total siRNA resulted in a significant reduction in cell viability to approximately 39% (p-value < 0.0001; Student's t-test).

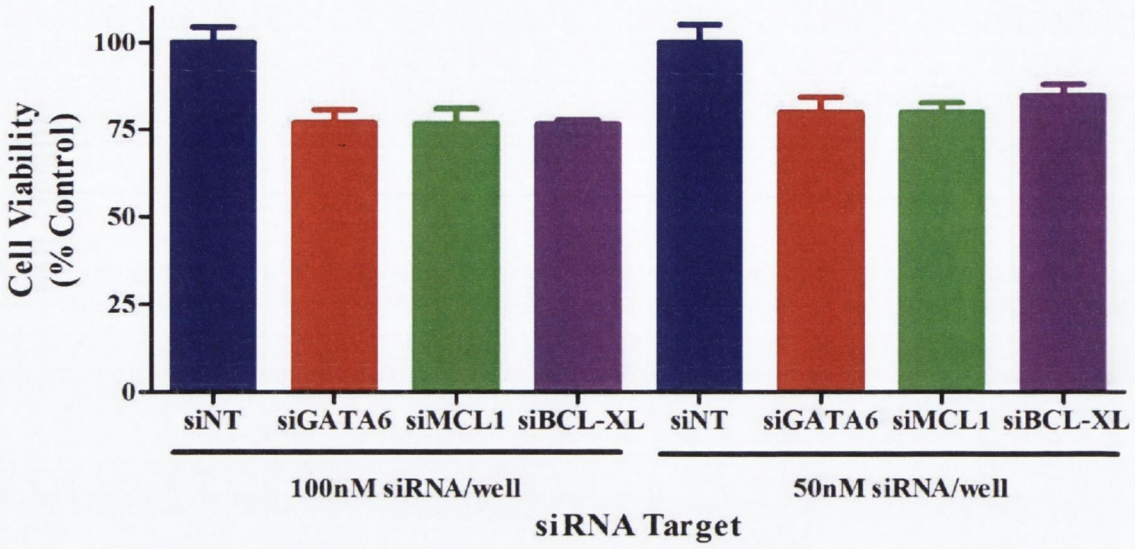


Figure 4.12: Cell viability of GOhTRT cells following specific inhibition of GATA6, MCL1 and BCL-XL using siRNA at 50nM and 100nM per well. The reduction in cell viability of GOhTRT cells following inhibition of GATA6, MCL1 and BCL-XL was not significantly altered using a final concentration of siRNA of 50nM as opposed to 100nM. The initial seeding density was 20,000 cells per well. (p-value > 0.05 for paired comparison of each siRNA at 50 and 100nM, Student's t-test; siNT: non-targeting control siRNA.)

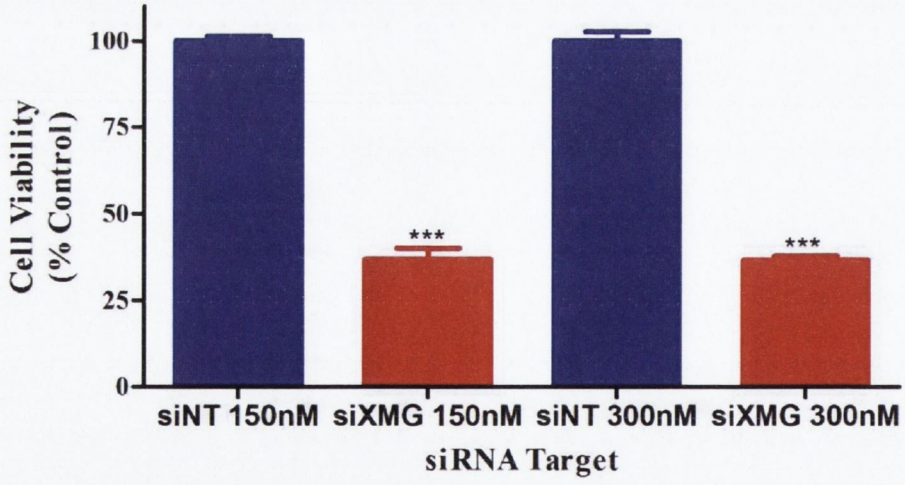


Figure 4.13: Cell viability of GOhTRT cells following the simultaneous inhibition of GATA6, BCL-XL and MCL1. The siRNA pools targeting BCL-XL (X), MCL1 (M) and GATA6 (G) were combined and the cell viability of the GOhTRT cells was assessed after 72 hours. The final concentration of siRNA per well was 150nM (each siRNA 50nM) or 300nM (each siRNA 100nM). Both final concentrations of siRNA resulted in a significant decrease in cell viability to 39% of control cells treated with non-targeting siRNA (siNT) at the appropriate concentrations. The initial seeding density was 20,000 cells per well. (*** p-value < 0.0001, Student's t-test; siNT: non-targeting control siRNA)

4.3.4 Optimisation of siRNA transfection conditions of GOhTRT cells

Using a chemical inhibitor screen, BCL-XL and BCL2 have been identified as modulators of resistance to DCA induced cell death in GOhTRT cells. BCL-XL and BCL2 are therefore candidates as positive controls for an siRNA library screen assessing apoptotic resistance in the GOhTRT oesophageal dysplastic cell line. Optimisation of siRNA transfection for this cell line is required prior to specific inhibition of BCL-XL and BCL2.

Optimal conditions for successful siRNA transfection vary between cell lines. Therefore, optimisation of a number of factors must be carried out for each cell line. These factors are the selection and quantity of transfection reagent to be used, final concentration of siRNA and quantity of transfection complex used per well (transfection reagent and siRNA in complex). Control siRNA complexes are available from Dharmacon™ (Thermo Scientific) that facilitates the optimisation of transfection conditions. Off-target effects resulting from suboptimal siRNA transfection conditions can be determined using a non-targeting control siRNA (siNT or NT). Efficiency of siRNA transfection conditions are assessed using siTOX (TOX or TX). This siRNA induces cell death, allowing transfection efficiency to be determined by the level of cell death.

Each of the four DharmaFECT reagents (D1, D2, D3 & D4; 0.4µl per well) were used to transfect siNT (100nM) and siTOX (100nM) into GOhTRTs. Cell viability was assessed using an MTT cell proliferation assay. The siRNA transfection was carried out using a reverse transfection method in 96 well tissue culture plates. Cells were seeded at 20,000 cell per well in 80µl of media with 20µl of transfection complex (transfection reagent in complex with siRNA) in each well prior to addition of the cell suspension.

This optimisation experiment demonstrated that siTOX in conjunction with DharmaFECT 4 resulted in the greatest reduction in cell death with cell viability reduced by ~80% (Figure 4.14). It was observed that the cell viability of GOhTRT cells treated with each of the DharmaFECT transfection reagents 1, 2, 3 and 4 in complex with siNT was substantial decreased to ~35%, ~65%, ~60% and ~60% respectively. This would indicate sub-optimal transfection conditions were used, resulting in toxicity caused by the transfection protocol. This is possibly due to an excess of transfection reagent in the transfection complex which has not bound with the siRNA. Therefore, the volume of DharmaFECT 4 used in each well was reduced in subsequent experiments. The off-target effect was not observed in wells which had the DharmaFECT 4 transfection reagent reduced from 0.4µl per well to 0.2µl per well (Figure 4.15A). In order to ensure that the transfection conditions being used were functional at a molecular level as well as at the phenotypic level, the knockdown in expression of BCL-XL was assessed followed specific inhibition of this gene by a targeted siRNA. The expression level of BCL-XL was assessed using real time PCR. The reduction in the quantity of DharmaFECT 4 reagent did not compromise transfection efficiency, as demonstrated by the percentage knockdown of BCL-XL mRNA expression following specific inhibition using siBCL-XL. BCL-XL mRNA expression was reduced by ~90% (Figure 4.15B). Optimal transfection efficiency for GOhTRTs was determined to be DharmaFECT 4 reagent at 0.2µl/well with a final siRNA concentration of 100nM.

4.4 Discussion

The overall aim of this body of work was to develop a functional siRNA screen that could identify novel therapeutic targets for the treatment of oesophageal malignancies. Of critical importance to the success of this type of study is a robust foundation based on a well-defined experimental design. The two most important elements in the experimental design of a high throughput siRNA screen are the assay design and the upscaling and

automation of the siRNA transfection protocol. Important elements of the assay design process include the selection of an appropriate endpoint assay that is relevant to the initial question and is also compatible with a high throughput system.

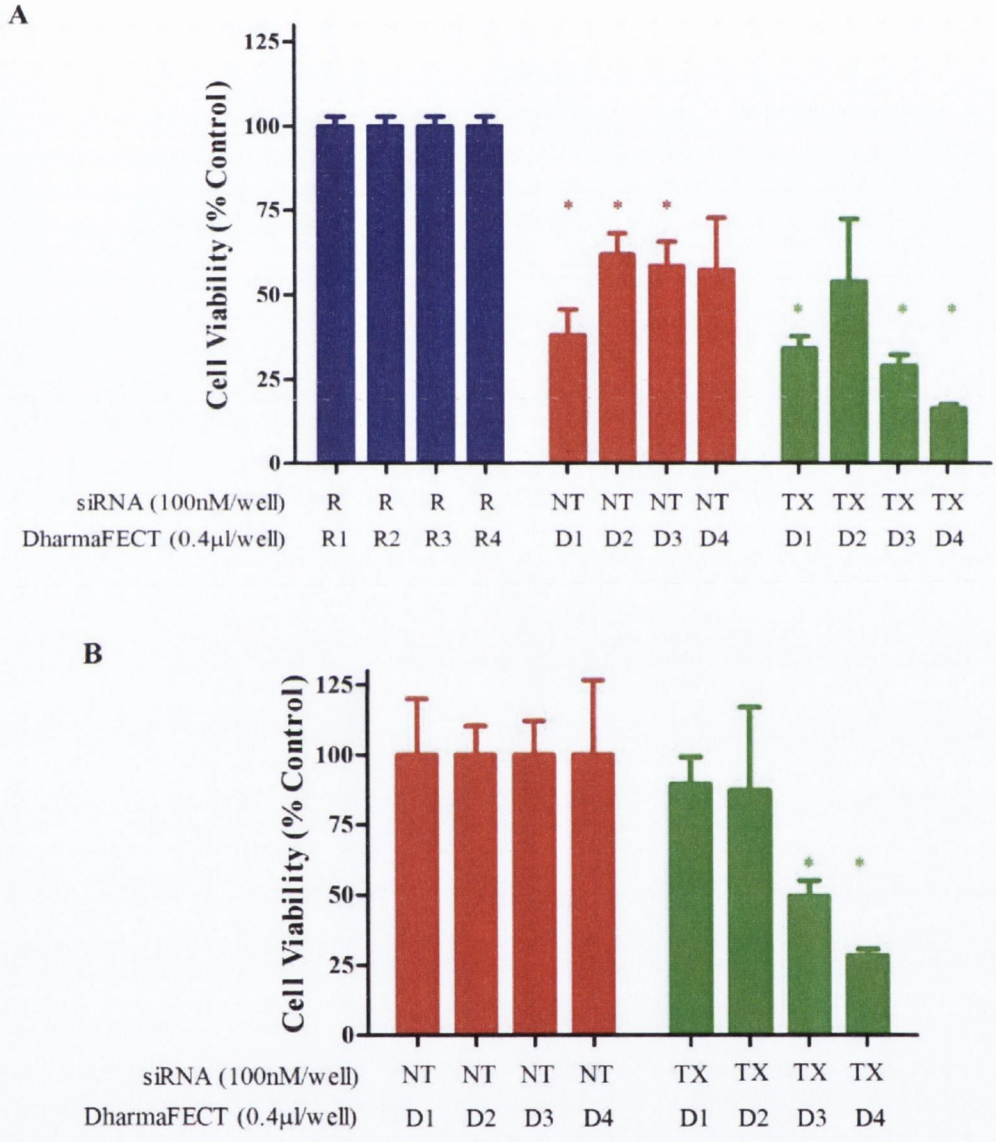


Figure 4.14: Optimisation of siRNA transfection conditions in GOhTRT cells. GOhTRT cells were transfected using complexes of DharmaFECT 1, 2, 3 or 4 (D1, D2, D3, D4) transfection reagent (0.4µl/well) with each of the control siRNAs, siNT or siTOX. When normalised to (A) non-treated resting controls and (B) siNT treated cells, the D4-siTOX complex reduces cell viability to the greatest extent. The reduction of cell viability following treatment with siNT indicates that there are substantial off-target effects under these transfection conditions and needs to be further optimised. (* = p-value < 0.05 compared to resting control, Student's t-test; NT: non-targeting control siRNA)

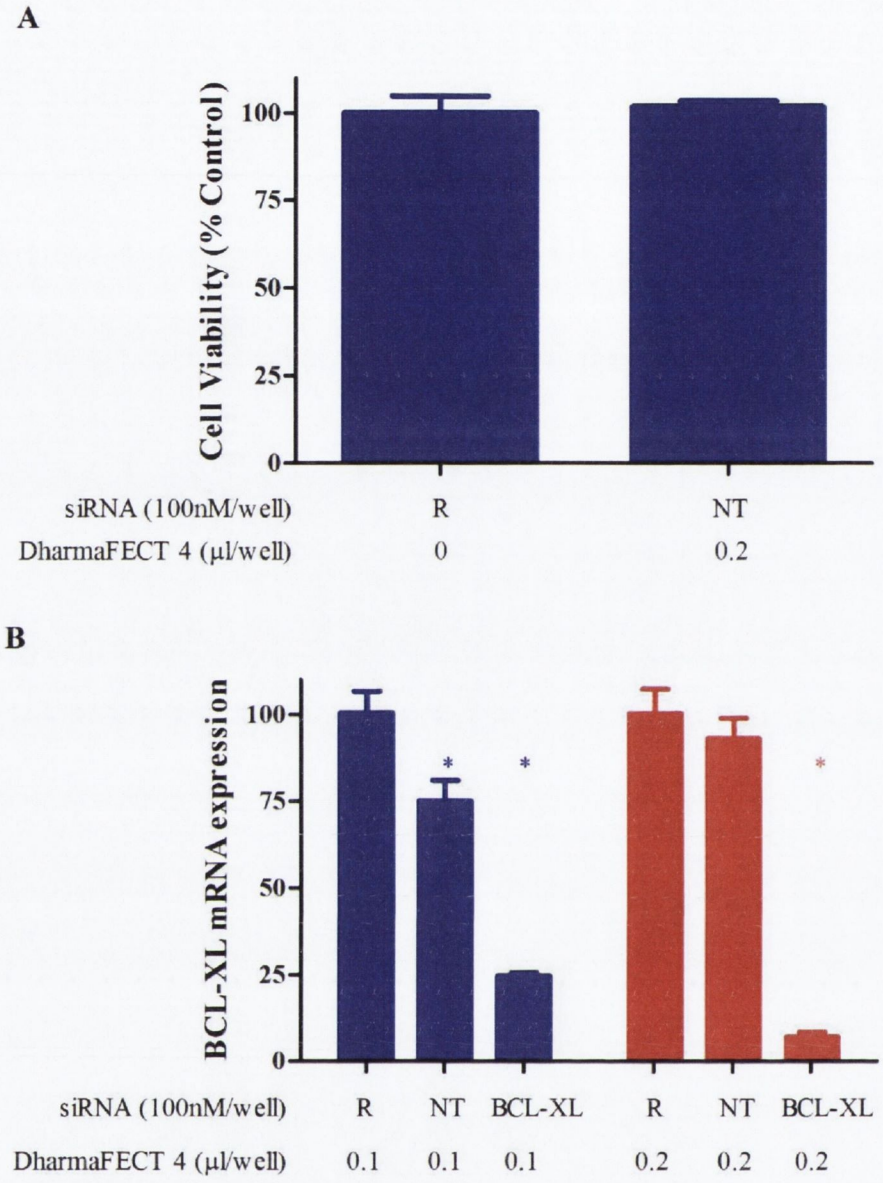


Figure 4.15: Reduction of the amount of DharmaFECT 4 transfection reagent reduces toxicity of transfection complex without a reduction in the potency of the siRNA knockdown. (A) A reduction in DharmaFECT 4 transfection reagent from 0.4µl/well to 0.2µl/well resulted in ablation of the off-target effect of the D4-siNT complex that resulted in cell toxicity. This reduction of transfection reagent did not reduce potency of siRNA inhibition, as demonstrated by (B) a greater than 90% knockdown of BCL-XL mRNA expression following treatment with an siRNA targeting BCL-XL. (* = p-value < 0.05 compared to resting control, Student's t-test; NT: non-targeting control siRNA.)

Primary siRNA screens such as the one under development in this chapter will often involve large numbers of targets. A balance must be achieved at this point in assay selection that facilitates the generation of sufficient data to answer the question under review without overwhelming the study with a huge quantity of data at this early stage of

the screening process. Ideally, the projected data generated per target should be inversely proportional to the number of targets under investigation. This would result in the larger, primary screens involving the least complex assays. The completion of secondary and tertiary screens would result in a decrease in the number of targets which facilitates an increase in the complexity of the assay (Figure 4.16).

Taking these factors into consideration, this body of work aimed to develop a functional siRNA screening system that is robust and reliable. This siRNA screen would be utilised to identify modulators of DCA signalling in oesophageal cell lines and cell proliferation in a dysplastic cell line in the absence of external stimuli. Based on the gene expression microarray data and systems biology analysis carried out previously, cell death and proliferation were identified as important processes related to a cohort of DCA responsive genes altered in OAC development. A cell proliferation assay was developed to examine the effects on the cell viability of oesophageal cell lines to DCA exposure and also to examine cell proliferation of a dysplastic oesophageal cell line. A semi-automated siRNA transfection protocol was developed which, in conjunction with the cell proliferation assay, provided a functional siRNA screening system that was applicable to both questions under examination. This screen would identify modulators of cell death in response to DCA and as such, potentially identify novel therapeutic targets in the treatment of oesophageal malignancies.

Ingenuity Pathways Analysis™ (IPA) software identified cell death and cell growth & proliferation among the highest ranked biological processes related to this cohort. As these cellular processes are also related to a number of established hallmarks of cancer (Hanahan and Weinberg, 2000), further investigations into the underlying mechanisms of these processes were undertaken. The viability of cell lines representing different stages of the

carcinogenic sequence was determined following exposure to DCA. Cell lines representing the end stages of the carcinogenic sequence (GOhTRT; SKGT4) were resistant to a DCA induced cell death. Normal squamous oesophageal cells (Het-1A) and metaplastic cells (QhTRT) displayed a significant reduction in cell viability (Figure 4.6).

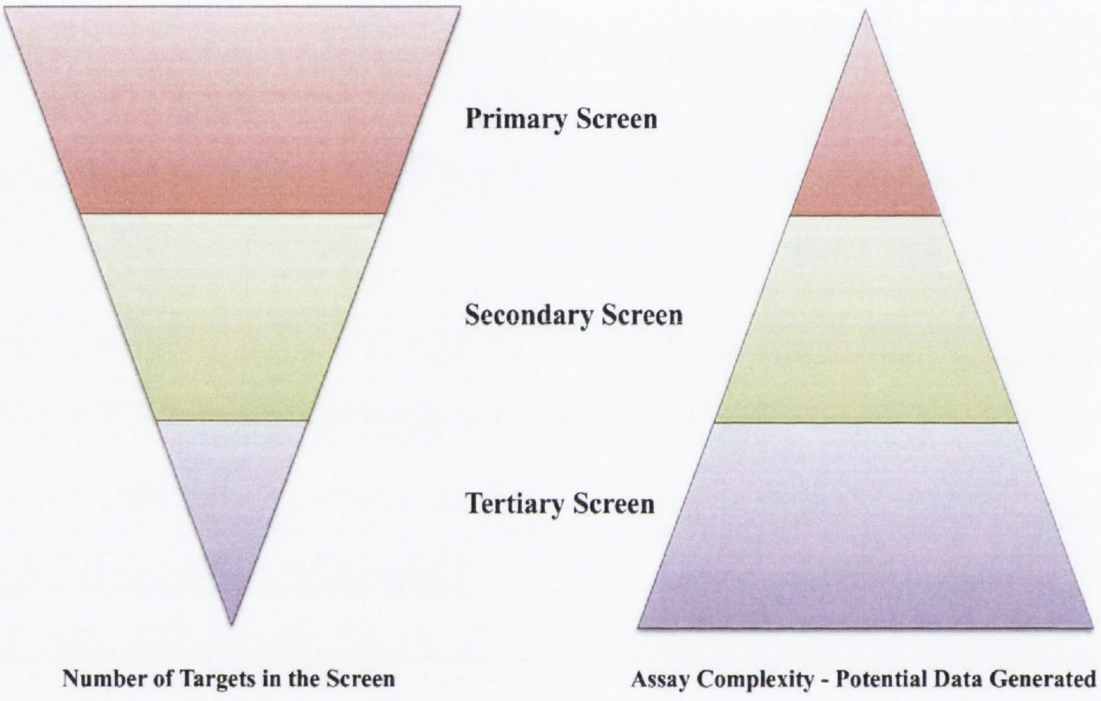


Figure 4.16: The inverse relationship between the number of initial targets in a screen to the complexity of the assay utilised. Primary siRNA screens traditionally aim to phenotypically characterise a large number of targets under particular experimental conditions. A subset of targets, identified as potentially interesting, will be selected and validated in subsequent screens. Following completion of each screen, the number of targets will be reduced due to the removal of false positives and false negatives. As the numbers decrease, the complexity of the assays used will increase to extract more information and confirm or disregard each of the hits. The quantity of data generated at each stage may remain similar but the reduction in initial targets will result in an increased volume of data relating to each individual target. This provides for a more informed decision on the validity of each target.

Van der Woude *et al* (2002) established that a switch to a more apoptotic resistant phenotype may occur during progression through the oesophageal adenocarcinoma sequence. Data presented in this study has clearly demonstrated that cell lines representing the end stages of adenocarcinogenesis indeed display a cell death resistant phenotype. Van der Woude *et al* (2002) also identified an increase in expression of the BCL-XL protein in

dysplastic and adenocarcinoma tissue. BCL-XL was therefore a prime candidate as a modulator of the observed resistance in GOhTRT and SKGT4 cells (van der Woude *et al.*, 2002).

The gene expression microarray study also revealed significant alterations in the gene expression profiles of a number of genes involved in oesophageal malignancy. Cell death and cell proliferation had also been highlighted in the microarray study as being involved in oesophageal carcinogenesis. A panel of gene targets were selected from this study, and also through a literature search for development as potential positive controls in an siRNA library screen. This library screen will assess the cell proliferation of a dysplastic cell line and attempt to selectively disrupt the proliferation and survival of these cells. The study would hope to identify potential novel therapeutic strategies to target dysplastic cells.

Development of oesophageal adenocarcinoma involves a number of well-established and documented pre-cancerous lesions known as the oesophageal carcinogenic sequence. As progression through each pre-cancerous lesion occurs, cells accumulate a greater number of mutations that confer advantageous characteristics to the cells to increase survivability and proliferative potential, important traits for cancerous cells. As cells transform, genetic instability increases, resulting in acquisition of further mutations beneficial to the malignant cell. Clones of cells with certain mutations such as loss of p16 or p53 or both have a selective advantage, as these cells continue to progress through the cell cycle unchecked regardless of the integrity of the genome of the cell. These mutations are generally resulting from loss of heterozygosity or sequence mutation/gene deletion and may occur apparently randomly (Maley, 2007). The development of adenocarcinoma from normal oesophageal cells occurs as a result of the accumulation of a number of genetic mutations which confer a competitive advantage on the transformed cells. The

transformed clones out compete the untransformed cells and allow for the development of disease. Identification of the genetic mutations that occur in transformed cells may facilitate the targeted disruption of transformed clones and the prevention of disease development. Cells with such beneficial mutations will out-compete non-mutated cells but the final events in the clonal evolution of metaplasia and invasion are not known. This body of work established that GOhTRT cells are more resistant to DCA induced cell death than normal oesophageal cells. As such, this cell line was developed as a model to investigate the molecular pathways that are involved in conferring resistance to DCA induced cell death in GOhTRT dysplastic cells, and possible identification of beneficial mutations in dysplastic cells. BO tissue has previously been suggested to exhibit resistance to DCA induced cell death (Dvorakova et al., 2005). The data described here has demonstrated a similar situation *in vitro* at the dysplastic stage of the oesophageal carcinogenic sequence and was subjected to further analysis.

GOhTRT cells represent the dysplastic stage of the oesophageal carcinogenic sequence. The extent of high-grade dysplasia correlates with the risk of developing adenocarcinoma (Buttar *et al.*, 2001). The GOhTRT cells have evolved from normal squamous cells and have acquired a resistance to DCA induced cell death. As such, GOhTRT cells were integrated into a model system to identify cellular pathways involved in DCA induced cell death. This model involved the inhibition of various cellular pathways using chemical inhibitors and siRNA. It may be possible, through identification of modulators of this phenotype and subsequent targeted inhibition of these modulators, to sensitise dysplastic cells to DCA induced cell death to a level similar to that of normal cells. This would be advantageous as cells resistant to bile induced cell death may have a selective advantage during an episode of reflux which would facilitate the development of a genetically unstable environment.

Commercially available chemical inhibitors are a useful tool for initial investigations into pathways and molecules that contribute to a particular phenotype. Indirect targeting using chemical inhibitors may explain the discrepancies that were observed between cell viability following chemical inhibition and specific inhibition by siRNA of the same target. Chemical inhibition of BCL2 in GOhTRT cells resulted in a decrease in cell viability when the cells were exposed to the BCL2 inhibitor at higher concentrations in conjunction with DCA (Figure 4.8D). Specific inhibition of BCL2 using siRNA did not sensitise GOhTRT cells to DCA induced cell death, even over an extended time course of ten hours. This was not the case with inhibition of BCL-XL. Chemical inhibition of BCL-XL resulted in a dose dependent sensitisation of GOhTRTs to DCA induced cell death which was mirrored using specific siRNA targeted inhibition of BCL-XL. The chemical inhibitor of BCL2, HA14-1, functions by disrupting BAX/BCL2 interaction, which allows the pro-apoptotic protein BAX, to induce caspase mediated apoptosis (Sigma Aldrich Data Sheet HA14-1). The chemical inhibitor of BCL-XL, BH3I-1, also targets the BH3 binding domain of BCL-XL. This prevents BCL-XL from inhibiting the action of the pro-apoptotic protein BAX (Sigma Aldrich Data Sheet BH3I-1). It is possible that sensitisation of GOhTRT cells to DCA induced cell death following inhibition of BCL2 at higher concentrations of the BCL2 inhibitor was a result of a non-specific effect by overloading the cell with inhibitor. Both the BCL-XL inhibitor and the BCL2 inhibitor target BAX binding sites. High concentrations of either inhibitor could result in non-specific binding to similar binding sites. As a result of the increased efficacy and specificity of siRNA inhibition, BCL-XL was identified as a modulator of resistance to DCA induced cell death in GOhTRT cells. BCL-XL was selected as a positive control in the development stages of a semi-automated siRNA screen to identify modulators of resistance to DCA induced cell death in GOhTRT using an siRNA library targeting GPCRs.

BCL-XL was further developed following on from this chemical inhibitor screen; however, a number of other interesting findings were uncovered that were not developed any further at this time. Functional NF-κB was identified as a critical factor in the survival of Het-1A cells to a much greater extent than in GOhTRT cells (Figure 4.8K&L). This may be a result of differential binding of NF-κB subunits between the cell lines but would require further investigation. Inhibition of MEK using the inhibitor PD98059 in GOhTRT cells co-treated with DCA resulted in an initial decrease in cell viability at low concentration followed by an increase in viability at higher concentrations of the inhibitor (>12μM).

BCL-XL is itself a promising candidate to develop into a therapeutic strategy for the treatment of oesophageal disease. A recent publication has described the development of novel small molecule inhibitors targeting the anti-apoptotic family proteins BCL2, BCL-XL and BCL-W. Clinical trials are currently underway involving one of these novel small molecule inhibitors that targets all three of the aforementioned members of the anti-apoptotic family of proteins. These clinical trials involve treatment of lymphoid malignancies, small-cell lung cancer and chronic lymphocytic leukaemia (Vogler *et al.*, 2009). That study demonstrates the inhibition of BCL-XL is a valid therapeutic strategy but may need to be inhibited as one element of a coordinated attack on more than one member of the anti-apoptotic family. The combination of BCL-XL, MCL1 and GATA6 inhibition in GOhTRT cells resulted in a reduction in cell viability by ~61% (Figure 4.13). These data agree with the hypothesis proposed by Vogler *et al* (2009) that the two subclasses of anti-apoptotic family members must be neutralised for apoptosis induction. BCL-XL and MCL1 represent each of the two subclasses. The targeted inhibition of siRNA may be a useful tool to combine with current employed strategies and methods to combat cancers where aberrant expression of anti-apoptotic proteins drives the development of tumours or confers resistance to current therapeutic drugs. The

identification of BCL-XL as a modulator of resistance to DCA induced cell death and a facilitator of cell proliferation in a dysplastic oesophageal cell line indicate a further possible application for the novel small molecule inhibitors described by Vogler *et al* (2009). The identification of GATA6 as a modulator of the proliferative potential of oesophageal cells is an important finding as this molecule has recently been linked with the regulation of canonical Wnt signalling. GATA6/Wnt signalling was implicated in maintaining the balance between progenitor cell expansion and differentiation (Zhang et al., 2008).

A semi-automated siRNA transfection protocol was developed using the Matrix Hydra II™ and the Matrix WellMate™ liquid handling robots. Liquid handling robots facilitate the up-scaling of routine experiments while maintaining the integrity of the assay. The accuracy and consistency of these liquid handling robots was assessed and deemed to be acceptable for inclusion in the semi-automated siRNA library screen. There were some inconsistencies observed within individual needles which were slightly higher than recommended. Following inspection, these needles were not found to have any visible mechanical faults that would contribute to these inconsistencies. Any attempted improvements in consistency were unsuccessful. The wells in question were recorded and positive targets from this area of the plates would be examined closely.

This section of work has developed an automated siRNA transfection protocol which can easily be adapted to incorporate a number of assays and cell lines. Both assays developed here utilised an MTT assay to assess cell proliferation. For example, substituting a fixation step for the MTT assay would facilitate an array of immunofluorescence experiments. There are numerous kits and immunofluorescent antibodies currently available to assess all characterised molecular signalling pathways. Automated fluorescent microscopy has

transformed high throughput analysis, increasing the number of parameters that can be evaluated in one well. Advances in sophisticated analysis software has facilitated the development of high content/high throughput screens. These screens can examine a number of parameters and generate huge quantities of data. As such, the semi-automated siRNA transfection protocol that has been developed is an important stepping stone in the development of more complex and comprehensive high content/high throughput screens.

Chapter 5

Results

The Identification of Modulators of Resistance to Deoxycholic Acid Induced Cell Death in a Dysplastic Oesophageal Cell Line following Implementation of an siRNA Screen Targeting a Library of G-protein Coupled Receptors

5 siRNA library screen results

5.1 Introduction

The development of oesophageal cancer has previously been described highlighting the stepwise fashion by which carcinogenesis occurs. The progression from the pre-malignant stage of Barrett's oesophagus (BO) to oesophageal adenocarcinoma (OAC) requires the acquisition of a number of traits which confer a selective advantage to the mutated malignant cells. These traits are commonly referred to as the hallmarks of cancer and were originally described by Hanahan and Weinberg (2000). The hallmarks of cancer are self-sufficiency in growth signals, insensitivity to anti-growth signals, evading apoptosis, sustained angiogenesis, limitless replicative potential and tissue invasion and metastasis (Hanahan and Weinberg, 2000). Recently, inflammation has been proposed as the seventh hallmark of cancer (Colotta *et al.*, 2009). Gene expression microarray data experiments have identified a number of cellular pathways and processes that are associated with a cohort of DCA responsive genes that are involved in the metaplastic-dysplastic-adenocarcinoma (M-D-A) sequence. These processes include cellular movement, cell-to-cell signalling and interaction, cellular growth and proliferation, cell death and the immune response. As demonstrated in the previous chapter, a dysplastic oesophageal cell line, GOhTRT, exhibits resistance to DCA induced cell death. An siRNA screen was developed to investigate the modulators of this resistance to DCA signalling.

Cell death or cell viability is an ideal process to investigate in a systematic siRNA screen. There are numerous assays available that are well characterised and robust which are adaptable to a high throughput system. The reliability of the chosen reporter assay, an MTT cell proliferation assay (LGC Standards, Middlesex, UK), coupled with the use of siRNA pools minimises the likelihood of false negative or false positive results. Off-target effects associated with oversaturation of the cellular machinery involved in siRNA

processing are countered by using a pool of siRNA molecules targeting four independent transcripts (Echeverri *et al.*, 2006). This accommodates the use of a lower concentration of individual siRNA molecules which reduces the chance of off-target effects. The screen to be completed is a primary screen and as such requires maximum detection sensitivity and maximum silencing of the genes (Echeverri and Perrimon, 2006). While every effort has been made to minimise the detection of false positive and false negative targets, the nature of high throughput screening will inevitably results in false results. The process of secondary and tertiary screens would aim to identify these false results and validate true results.

In the context of this study, which aims to assess resistance to DCA induced cell death in GOhTRT cells, the siRNA transfection protocol that has been developed was used in an siRNA library screen targeting G-protein coupled receptors (GPCRs). A library targeting GPCRs were chosen as these molecules represent 15% of the druggable genome and 25% of the current viable experimental and marketed drugs (Hopkins and Groom, 2002). GPCRs have also been linked with bile acid signalling (Kawamata *et al.*, 2003). Of the cell lines tested, the oesophageal dysplastic cell line, GOhTRT was selected for further investigation in the siRNA library screen as this cell line demonstrated resistance to DCA induced cell death (Figure 4.6). The identification of modulators of resistance to apoptosis in dysplastic cells could lead to the identification of novel therapeutic strategies in the prevention of the development of oesophageal adenocarcinoma.

Systematic siRNA library screens have previously been published which have identified modulators of resistance to current chemotherapeutic treatments (Giroux *et al.*, 2006) and novel therapeutic targets (Tyner *et al.*, 2009). The primary aim of this body of work was to

carry out an siRNA library screen to identify modulators of resistance to cell death in GOhTRT cells using a GPCR library.

5.2 Objectives

A semi-automated siRNA transfection protocol has been developed (Section 4.3.1). A high throughput cell proliferation assay has also been developed using an MTT assay. This section of work aims to:

- complete an siRNA library screen targeting GPCRs in GOhTRT cells.
- critically assess the quality of the data produced by the siRNA library screen.
- identify modulators of resistance to DCA induced cell death in GOhTRT cells.

5.3 Results

An siRNA library screen targeting G-protein coupled receptors (GPCRs) was carried out using the previously developed semi-automated siRNA transfection protocol. The aim of this siRNA library screen was to identify modulators of resistance to DCA induced cell death in GOhTRT cells. Viability of GOhTRT cells following siRNA inhibition and DCA exposure (10 hours; 500μM) was assessed using an MTT cell proliferation assay. An siRNA targeting BCL-XL was incorporated as a positive for sensitisation of GOhTRT cells to DCA induced cell death. A non-targeting siRNA (siNT) was used as a negative control.

5.3.1 Quality Assurance/Quality Control of siRNA screen

5.3.1.1 Graphical representation of statistical performance of screen

The siRNA libraries were supplied by Dharmacon and contained siGENOME siRNA pools. Each pool is comprised of 4 siRNA molecules independently targeting non-overlapping regions of the mRNA of the gene of interest in each well. The GPCR siRNA library is comprised of 516 siRNAs pools. This library was available in a 96 well format

with one target per well which required seven plates to accommodate all siRNAs. There were 80 siRNA pools per plate which left two columns of eight wells (columns 1 & 2 of the 96 well plate) available for assay specific control siRNAs. Each plate (n=7) was replicated four times for this screen giving a total of 28 plates. Each replicate was plotted in a box and whiskers graph to display the median and interquartile ranges of each plate. The whiskers were derived using the tradition Tukey method where each whisker represents 1.5 times the interquartile range or terminates at the last datum point if that results in shorter whiskers (Figure 5.1). This graphical representation displays the descriptive statistics of each replicate plate in a more easily interpretable fashion than examining raw data.

The box plots representing the replicates of plate one do not appear to be as consistent as the other plates. The median values range from ~85% to ~110%. Replicate 1 also has a number of outliers at the lower end of distribution which are absent on the other replicates. The median and range of distribution of the replicates of the other plates are more consistent. It is inconclusive from this analysis if one or more of the replicates of plate 1 are defective. Therefore, the decision was made to include all replicates in future Quality Assurance/Quality Control assessments.

5.3.1.2 Scatterplots comparing each replicate of each plate

Similarities between each replicate plate were visualised using scatter plots. These scatterplots were obtained by plotting each replicate plate against each of the other replicates for that particular plate. Each value is represented by a single dot on a scatterplot. A high level of similarity between two replicates would result in all values clustering along a diagonal line from the origin with a slope of 1. This type of plot also allows for statistical representation of the similarities between the two sample datasets. In

this case, the Spearman correlation coefficient value was used to determine the goodness of fit of the two replicates (Figure 5.2). The Spearman correlation coefficient value for each replicate plotted against every other replicate is summarised in Table 5.1.

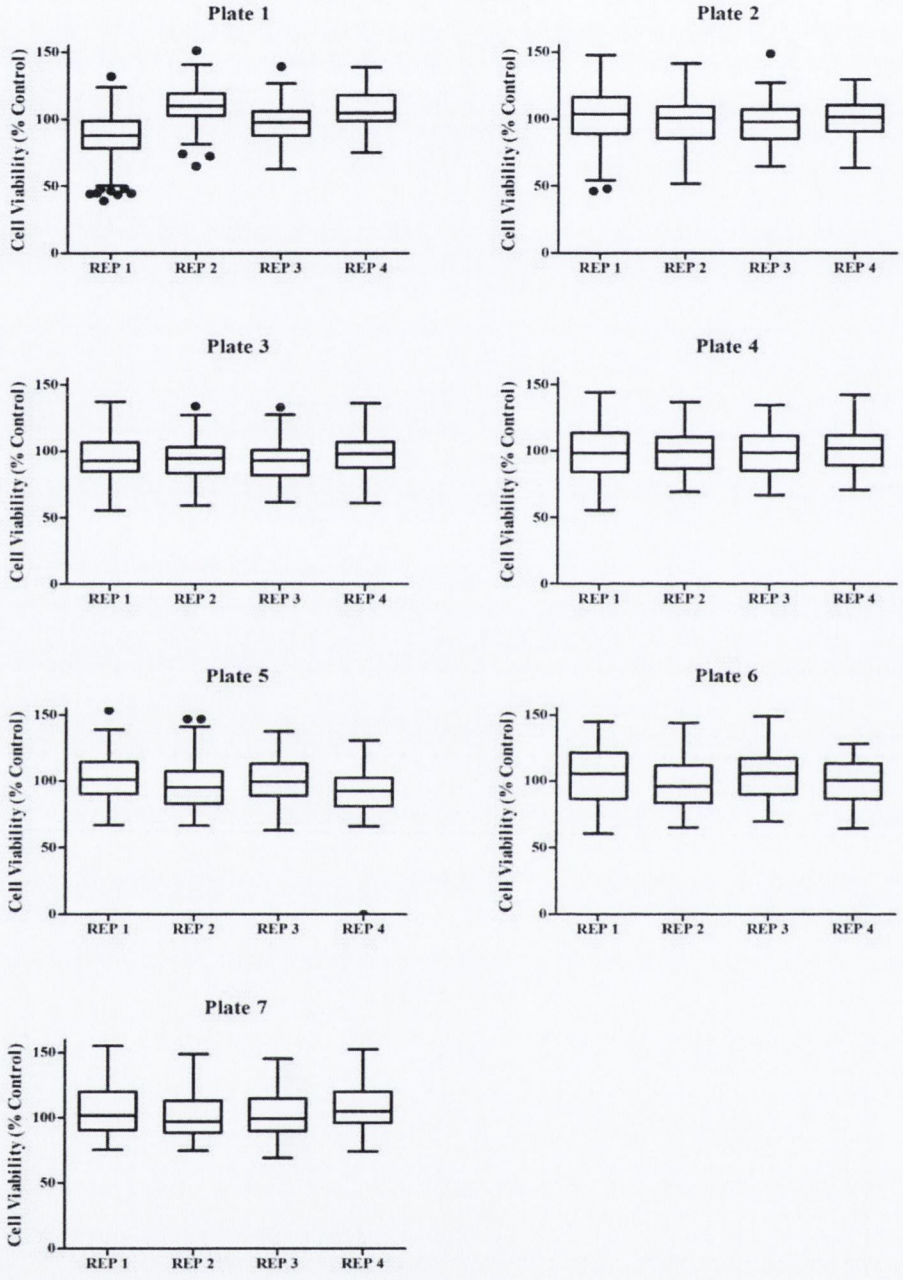


Figure 5.1: Box and whisker plots of all replicates of all plates in the GPCR siRNA library screen. A box and whisker plot of each replicate is a graphical representation of the descriptive statistics of the data generated by the siRNA screen. The median, the range of distribution and the presence of outliers are represented on this type of graph. The whiskers are derived using the traditional Tukey method where each whisker represents 1.5 times the interquartile range or terminates at the last datum point if that results in shorter whiskers. Filled circles represent values outside this range. The replicates of plate one appear to be inconsistent with a range in median values from ~85% to ~110%. The replicates of the other six plates are more consistent.

Table 5.1: Summary of Spearman correlation coefficient values for each replicate of each plate.

	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	Plate 7
Rep 1 v Rep 2	0.5345	0.7008	0.7397	0.7911	0.8103	0.2323	0.8454
Rep 1 v Rep 3	0.6485	0.6101	0.7623	0.7695	0.7798	0.2404	0.7985
Rep 1 v Rep 4	0.1709	0.6562	0.6144	0.7669	0.7033	0.05578	0.6991
Rep 2 v Rep 3	0.5187	0.7705	0.7835	0.9199	0.8266	0.8617	0.8248
Rep 2 v Rep 4	0.02821	0.8099	0.7464	0.903	0.7856	0.8358	0.7203
Rep 3 v Rep 4	-0.08298	0.8121	0.6829	0.9006	0.719	0.8393	0.8261

Plates 2, 3, 4, 5 and 7 exhibit a high level of correlation between the replicates with Spearman correlation coefficient values greater than 0.6. This Spearman value demonstrates a good level of correlation. Comparisons involving replicate 4 of plate one have extremely low Spearman correlation coefficient values of less than 0.2. Replicate 1 of plate 6 does not appear to correlate with the other replicates of this plate as the Spearman correlation coefficient is less than 0.25. Box plots of the replicates of plate 1 indicate that replicate 1 has a different distribution than the other replicates including a cluster of outliers at the lower end of the distribution. These data suggest that there is a potential systematic bias involving the first replicate of each plate. In order to avoid the introduction of error into the system, the first replicate was removed from the analysis for each plate.

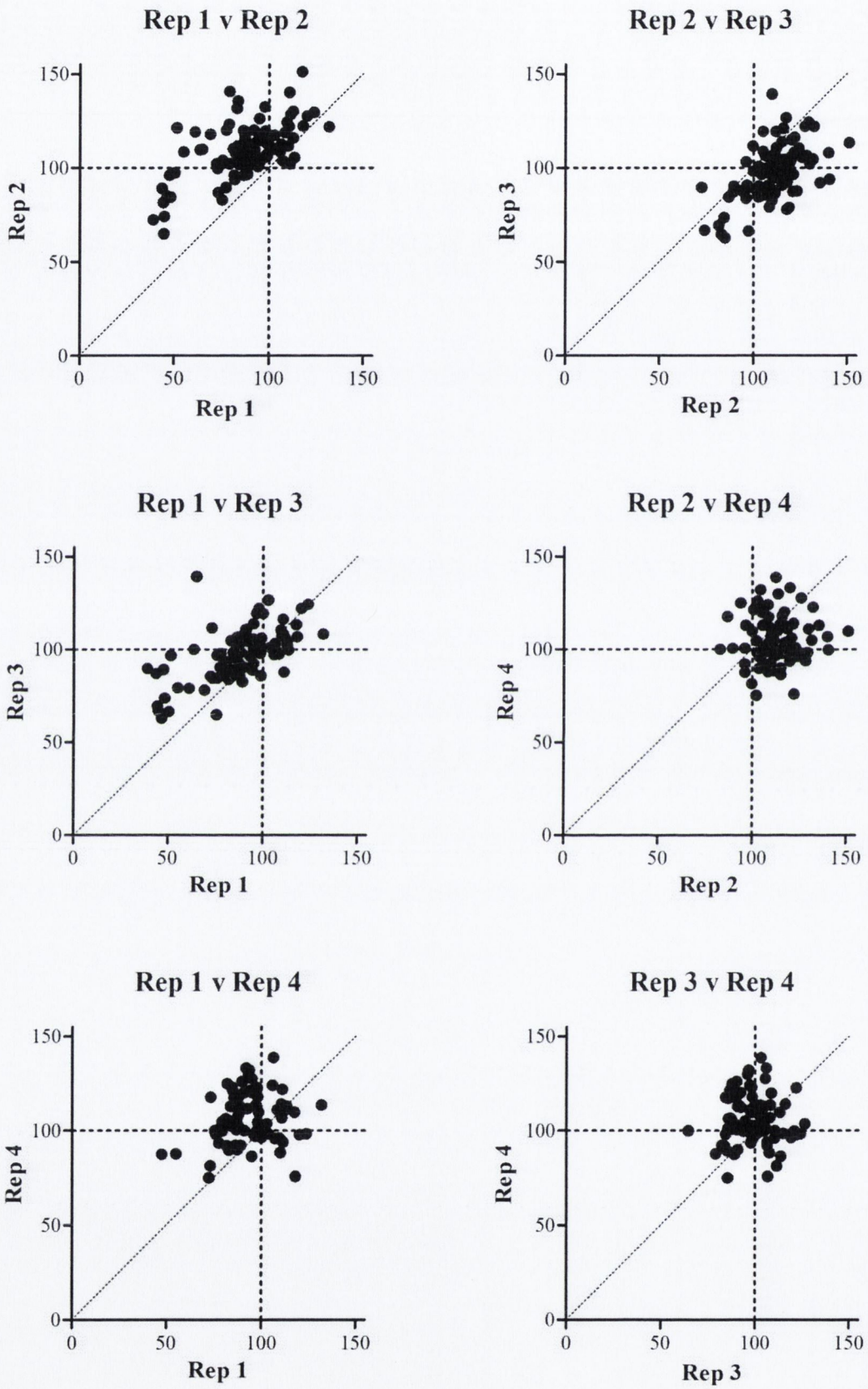


Figure 5.2A: Scatterplots displaying replicates of plate 1.

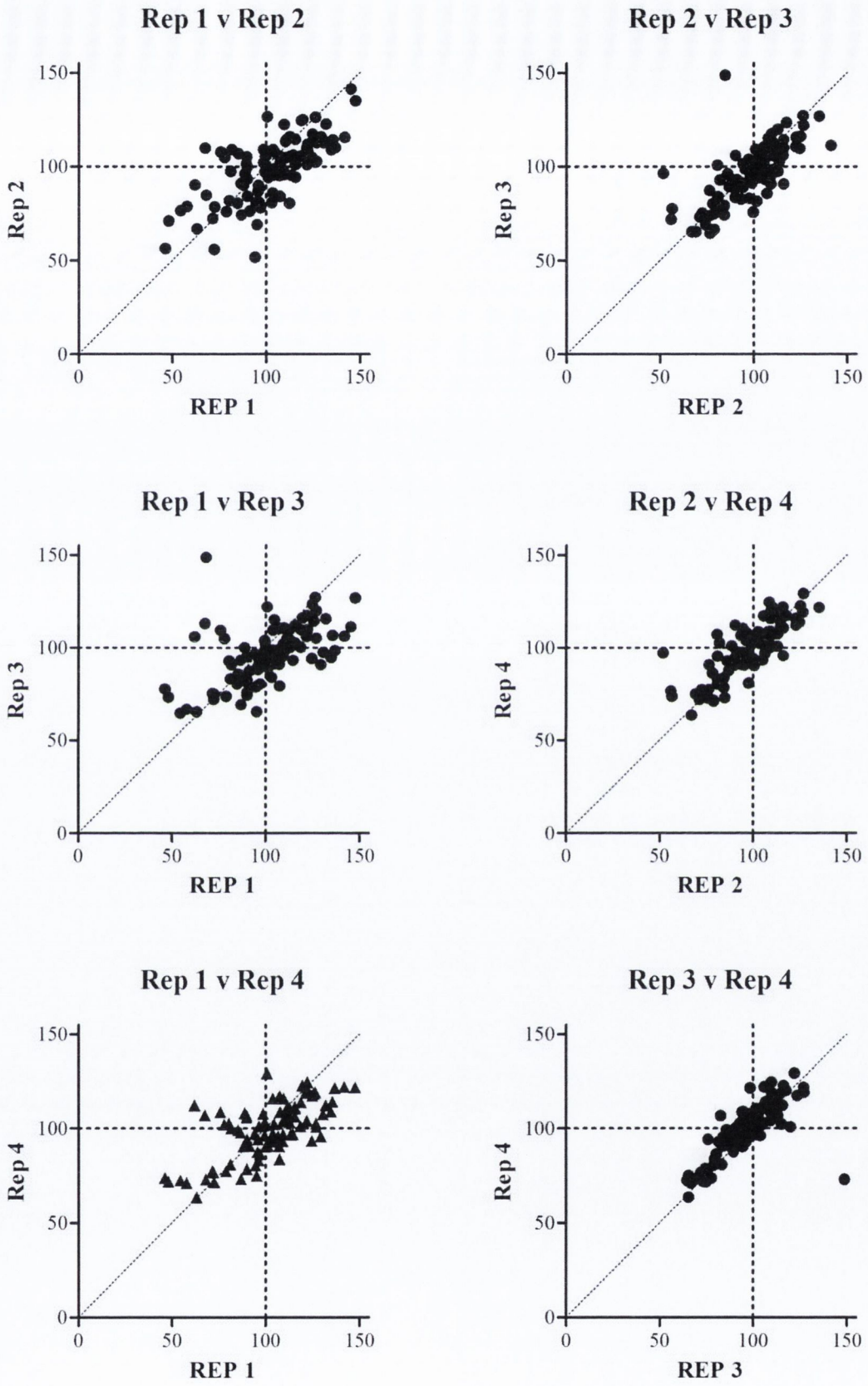


Figure 5.2B: Scatterplots displaying replicates of plate 2.

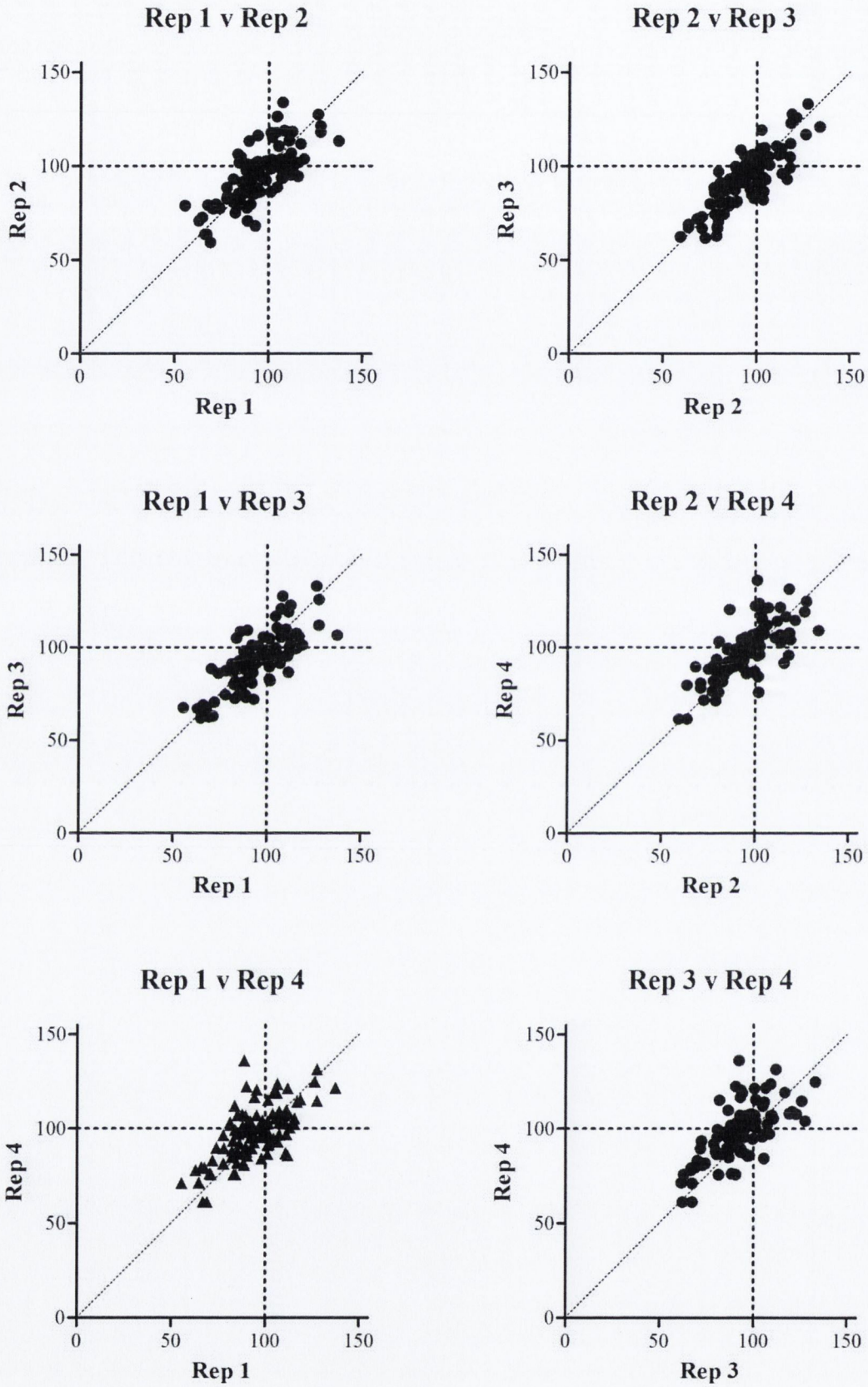


Figure 5.2C: Scatterplots displaying replicates of plate 3.

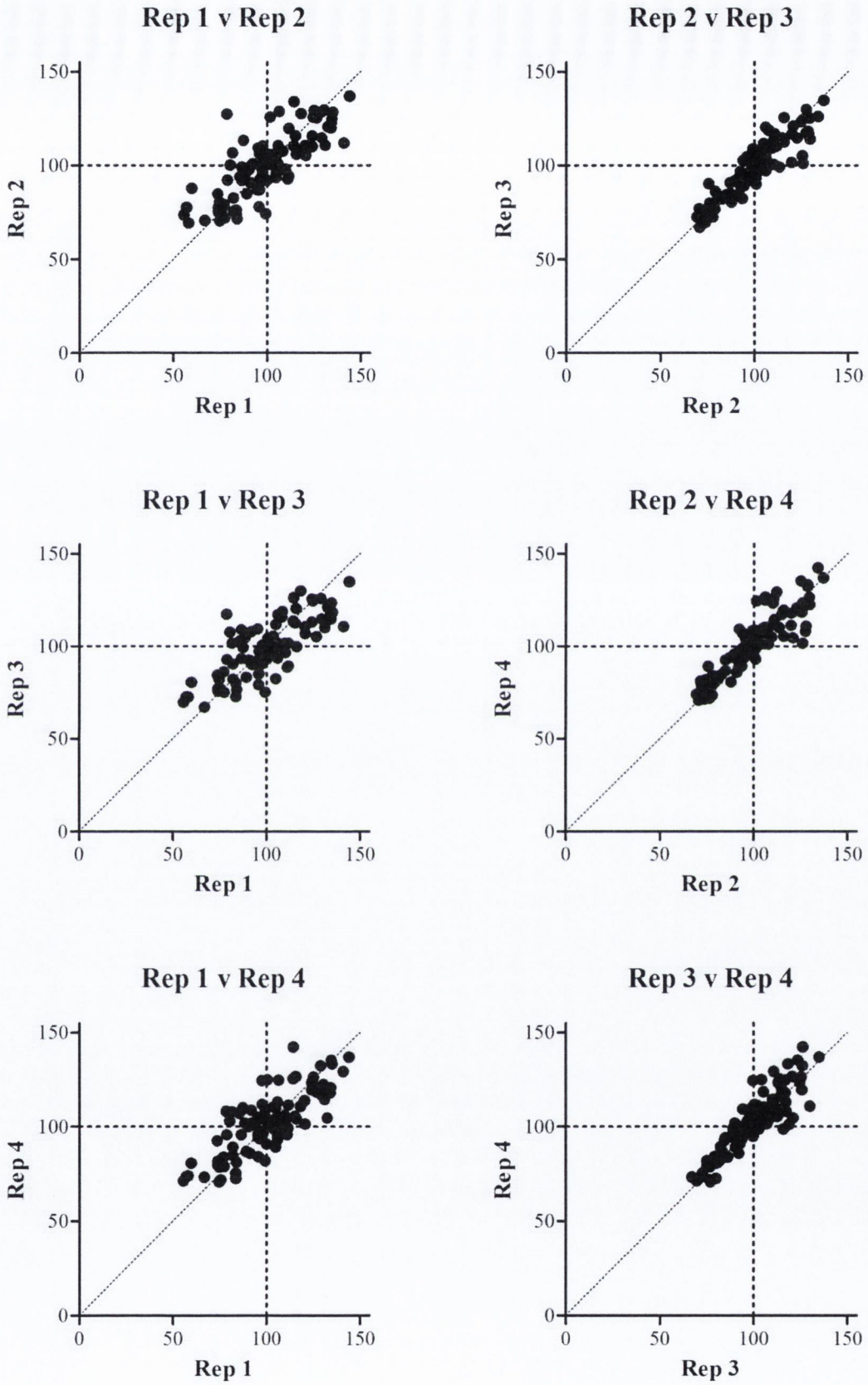


Figure 5.2D: Scatterplots displaying replicates of plate 4.

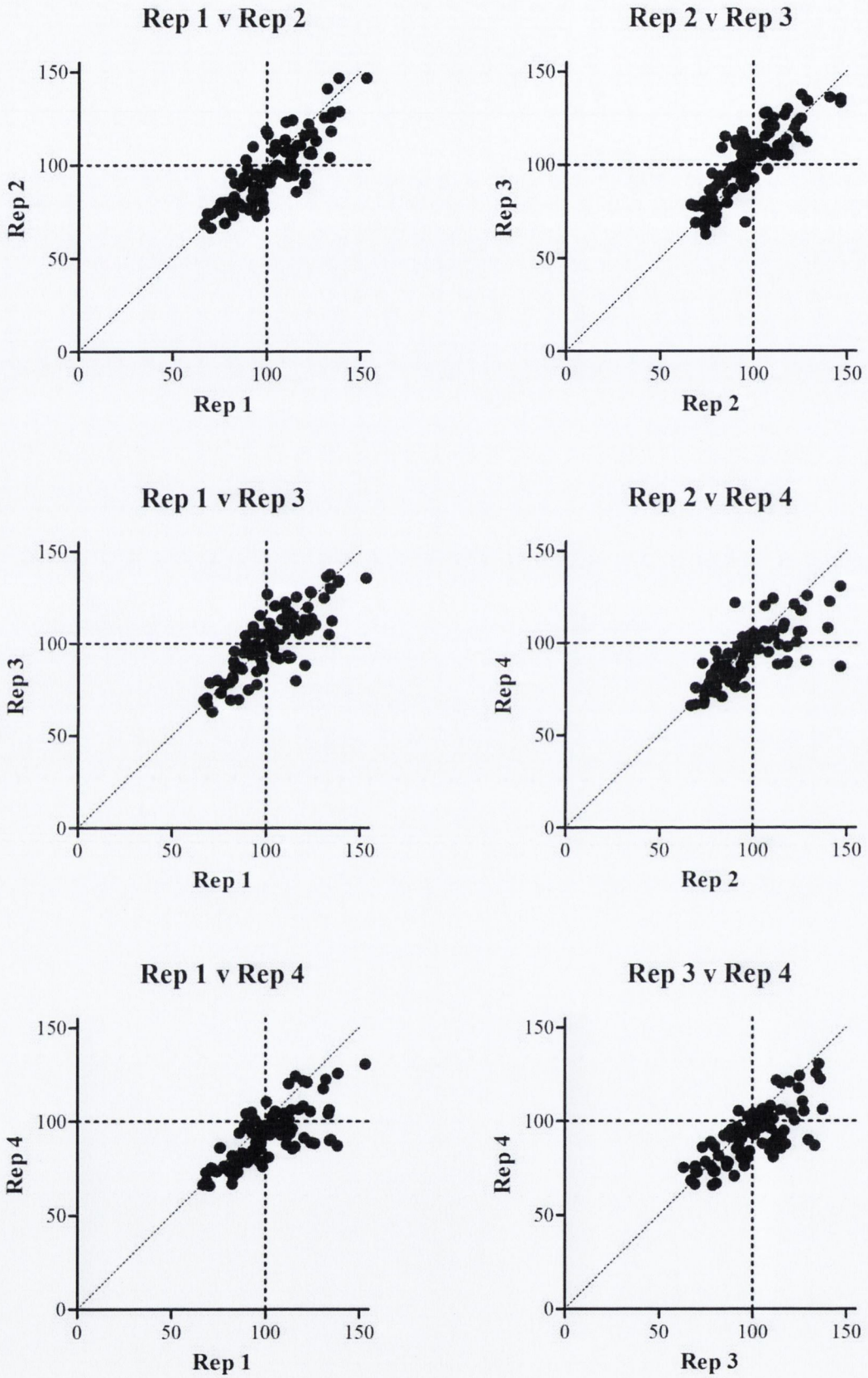


Figure 5.2E: Scatterplots displaying replicates of plate 5.

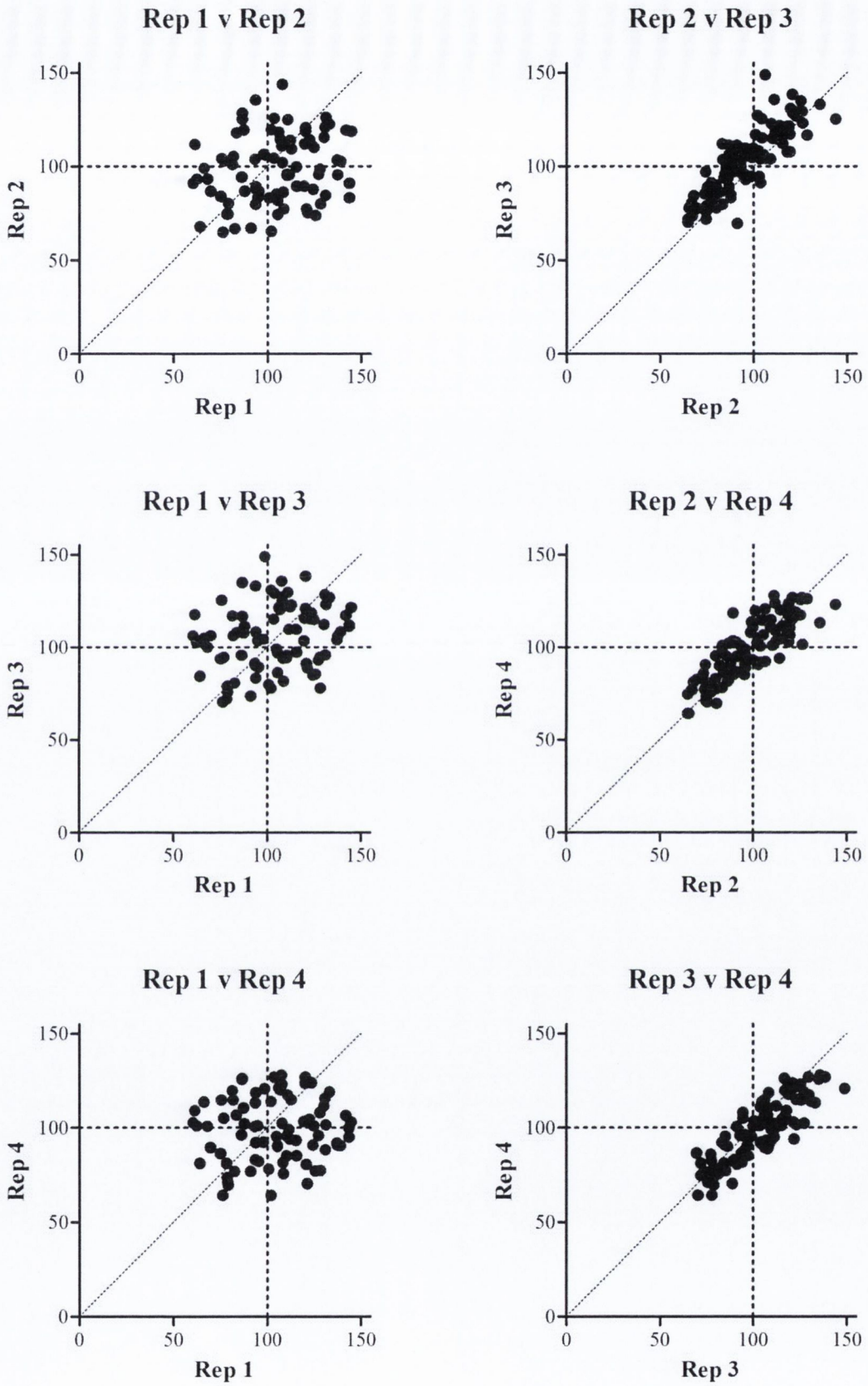


Figure 5.2F: Scatterplots displaying replicates of plate 6.

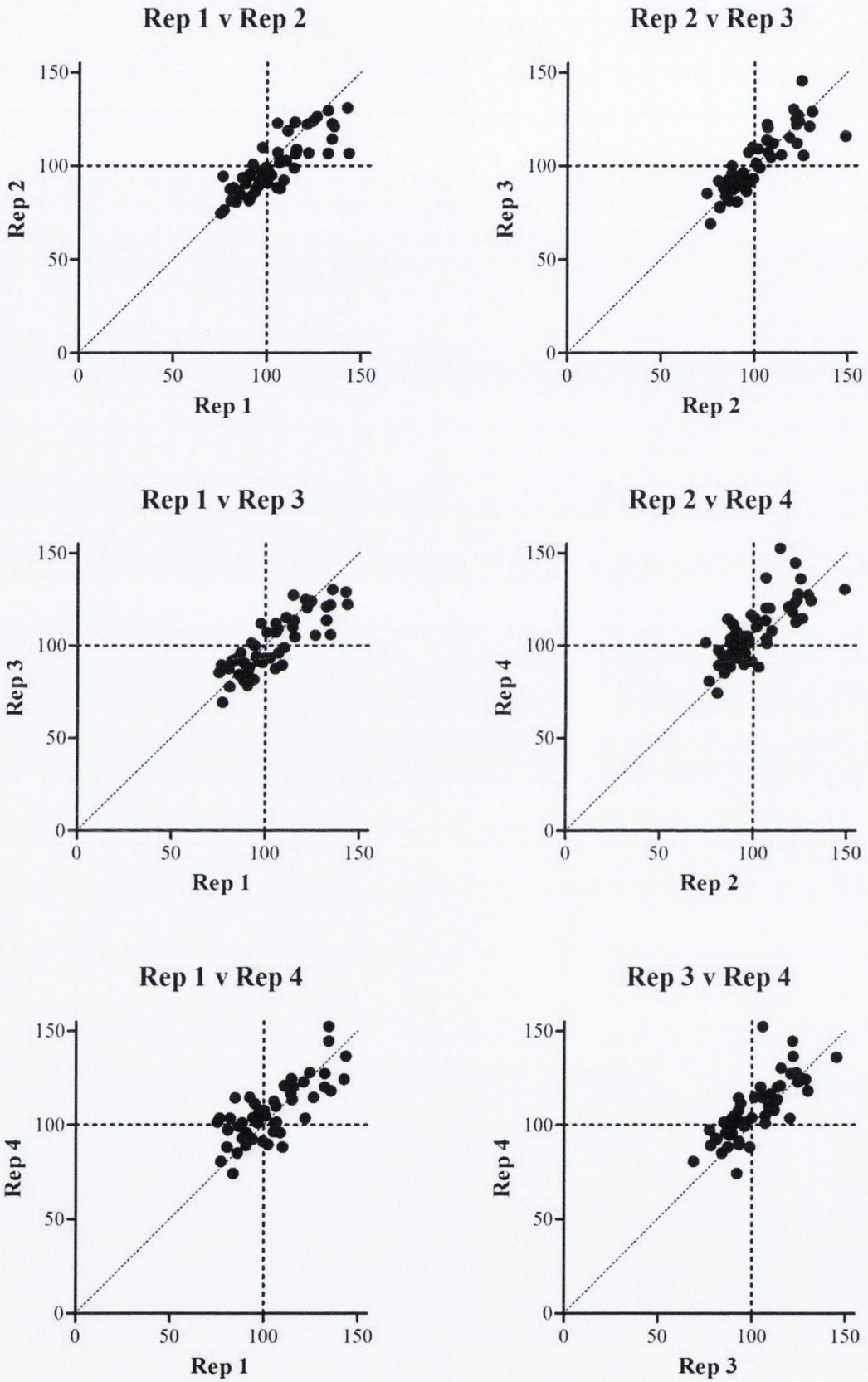


Figure 5.2G: Scatterplots displaying replicates of plate 7.

5.3.2 Top siRNA gene targets that sensitised GOhTRT cells to DCA induced cell death as ranked by cell viability

The aim of this siRNA screen was to identify modulators of resistance to DCA induced cell death in GOhTRT cells. An siRNA gene target involved in the mediation of the observed resistance to DCA apoptosis was deemed to be a positive target if inhibition resulted in a significant reduction (p-value < 0.05) in cell viability following exposure to DCA. Cell viability was calculated using the following formula: $\frac{abs\ of\ target\ well}{median\ abs\ of\ neg\ control\ well} \times 100$. The siRNA gene targets were ranked based on cell viability expressed as a percentage of the non-targeting siRNA controls. The top 20 targets are illustrated in Figure 5.3. A list of the top 50 targets ranked by cell viability is available in Table 5.2. The range of values of cell viability for the top 20 targets ranked according to cell viability was 61% to 82%. All of the top 50 targets and the positive controls were reduced to a statistically significant level when compared to negative controls (p-value < 0.05, Student's t-test).

5.3.3 Top targets of the siRNA library screen inhibiting a library of GPCRs in GOhTRT cells ranked according to z-score

The z-score is representative of the distance in units of standard deviation that the value in question is from the mean of the sample. The z-score is calculated using the following formula: $z = \frac{x-\mu}{\sigma}$; where x = sample value; μ = sample mean; σ = standard deviation. The z-score indicates how many standard deviations a value is above or below the mean. A negative z-score indicates that the value in question is less than the sample mean. Conversely, a positive z-score indicates that the value is greater than the sample mean. All siRNA gene targets, including negative and positive controls are displayed in Figure 5.4. The siRNA gene targets were ranked on z-score with the highest ranked target having the

lowest z-score (Figure 5.5). The range of z-score values among the top 50 positive targets was from -2.35619 to -1.01202. A list of the top 50 targets ranked by both cell viability and z-score is available in Table 5.2.

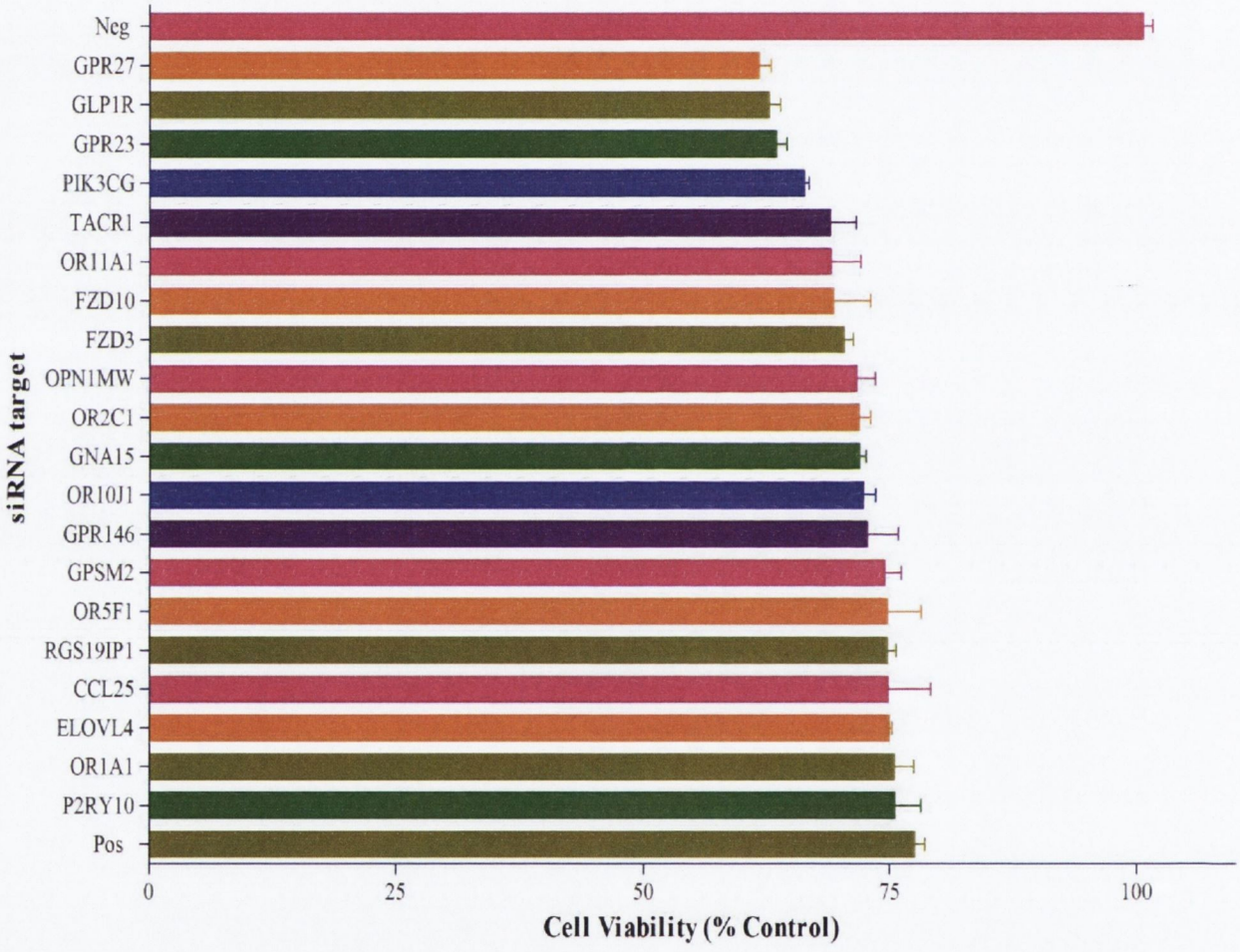


Figure 5.3: Top 20 targets of a GPCR siRNA library screen ranked according to cell viability. Targets were ranked based on the percentage cell survival of the GOhTRT cells following DCA exposure (10hrs; [500μM]) in conjunction with specific inhibition of each target gene using siRNA from a GPCR library. The range of values of the top 20 positive targets ranked according to cell viability was from 61% to 76%. All targets and positive controls illustrated were statistically significant compare with the negative control (p-value < 0.05 according to Student's t-test).

Table 5.2: Ranking of the top 50 positive targets according to cell viability and z-score.

	Cell Viability			z-score	
Gene Symbol	Rank	% Cell Via	p-value*	Rank	z-score
GPR27	1	61.7152962	3.63E-05	1	-2.44586
GLP1R	2	62.72577997	0.000103	3	-2.29774
GPR23	3	63.48364279	5.03E-05	2	-2.31759
PIK3CG	4	66.32213609	1.12E-07	4	-2.0165
TACR1	5	68.99224806	9.29E-07	5	-1.85285
OR11A1	6	69.01763224	7.18E-07	7	-1.78896
FZD10	7	69.29392447	0.000766	6	-1.83891
FZD3	8	70.36124795	0.00062	8	-1.7605
OPN1MW	9	71.62048699	4.18E-07	11	-1.6161
OR2C1	10	71.87237615	2.35E-07	12	-1.59679
GNA15	11	71.92118227	0.000918	9	-1.64824
OR10J1	12	72.29219144	2.93E-07	13	-1.57047
GPR146	13	72.67904509	0.000479	10	-1.63365
GPSM2	14	74.45255474	4.05E-05	14	-1.56881
OR5F1	15	74.76313523	1.15E-05	16	-1.49191
RGS19IP1	16	74.76313523	1.5E-06	18	-1.48262
CCL25	17	74.80842912	0.000241	24	-1.37255
ELOVL4	18	74.9589491	0.002083	20	-1.43404
OR1A1	19	75.48278757	1.6E-06	26	-1.3526
P2RY10	20	75.53832903	6.96E-06	21	-1.43256
HTR3A	21	75.66909976	8.72E-05	15	-1.49909
IL8RA	22	75.83130576	7.08E-05	17	-1.48882
GPR97	23	76.39902676	6.29E-05	19	-1.4525
LPHN3	24	76.5743073	7.15E-07	29	-1.28642
PNR	25	76.74418605	3.03E-06	25	-1.36027
GNA14	26	76.76518883	0.003683	28	-1.30833
HTR5A	27	77.29115977	8.07E-05	22	-1.39785
GPRC5C	28	77.69667478	9.68E-05	23	-1.37402
GPR155	29	77.91461412	0.00765	33	-1.22976
MGC26856	30	78.08564232	3.77E-05	35	-1.19744
HTR7	31	78.34549878	0.000136	27	-1.33491
FLJ31393	32	78.65353038	0.007513	37	-1.17636
PIK3CB	33	78.72523686	6.96E-05	31	-1.24229
OR2W1	34	78.75734677	2.71E-06	38	-1.14431
PTGFR	35	78.81136951	1.13E-05	34	-1.22038
GRM4	36	79.15652879	0.000279	30	-1.28148
OPN1SW	37	79.59697733	7.56E-06	43	-1.09066
GPR18	38	79.66401415	0.001982	40	-1.10287
OPRL1	39	79.68094039	8.69E-05	47	-1.07219
OR5I1	40	79.7588286	0.0008	36	-1.18656
CXCR4	41	79.80295567	0.02781	50	-1.04933
GPR10	42	79.84084881	0.002453	42	-1.09519
OR1D4	43	80.35264484	7.29E-06	-	-
P2RY8	44	80.36175711	0.044638	39	-1.13041
GPR3	45	80.54818744	0.002894	-	-
GNG3	46	80.81343943	0.003168	-	-
FZD1	47	80.95238095	0.013255	-	-
PTPN6	48	81.05081826	1.71E-05	-	-
GPR123	49	81.52077807	0.003264	-	-
OPRM1	50	81.69605374	0.000313	-	-
TAS2R41	-	-	-	32	-1.23922
HRH1	-	-	-	41	-1.10255
PTPN6	-	-	-	44	-1.08474
GPR68	-	-	-	45	-1.0782
HTR1B	-	-	-	46	-1.07249
OXER1	-	-	-	48	-1.06774
IL8	-	-	-	49	-1.05743

* compared to siNT negative control; Student's t-test

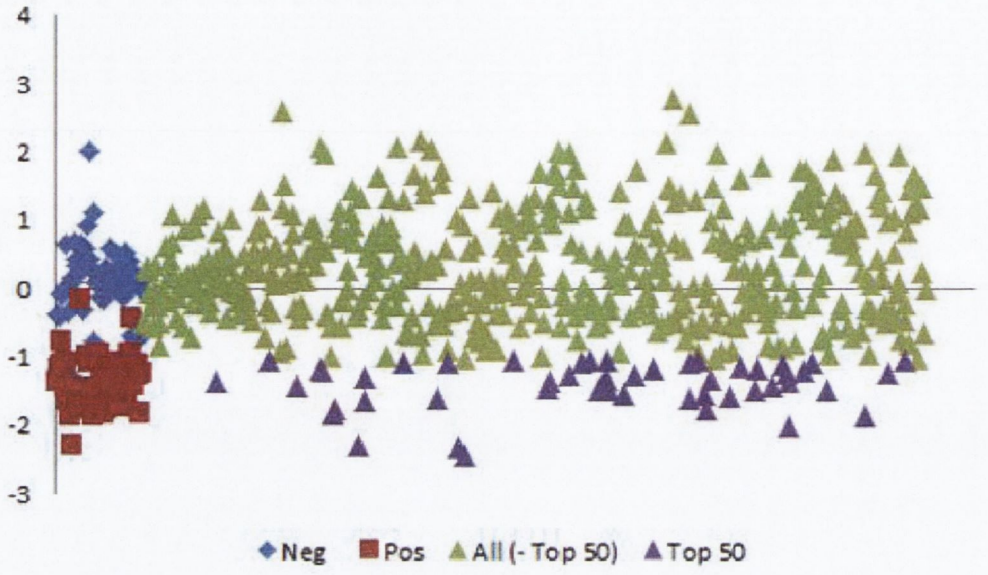


Figure 5.4: Scatterplot illustrating z-score for all siRNA target genes. The z-score was determined for each siRNA target gene in the screen including positive and negative controls. Positive and negative controls clustered together in distinct groups. The z-score of the siRNA gene targets in the GPCR siRNA library were distributed from a z-score of -2.5 to 2.9.

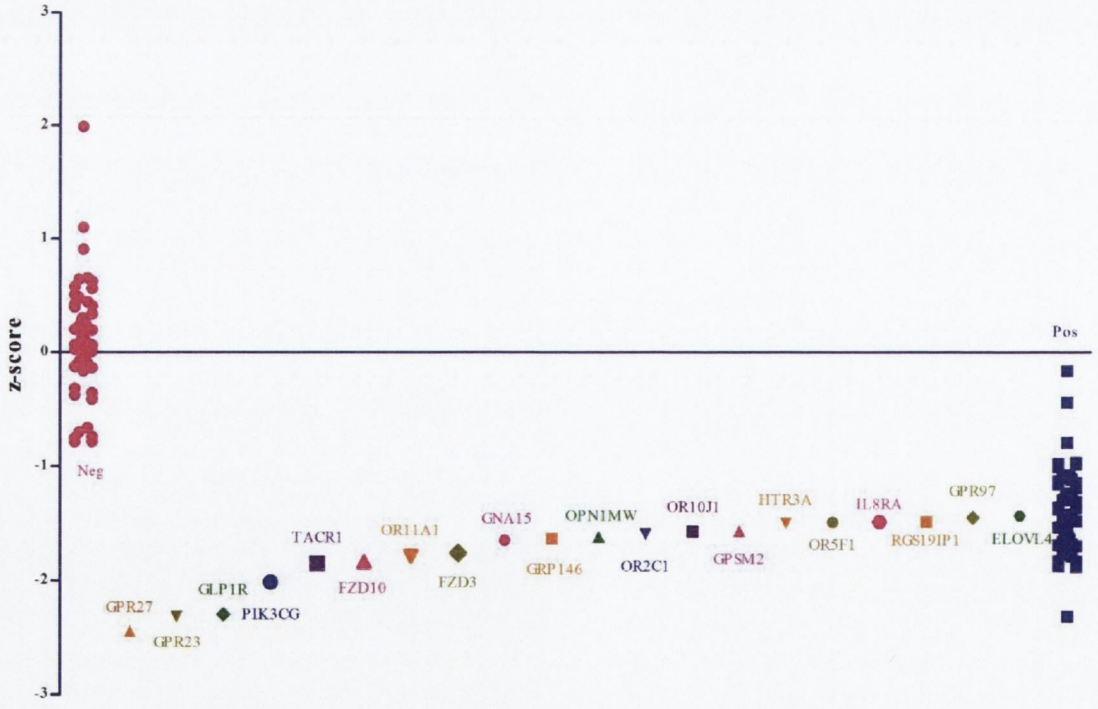


Figure 5.5: Top 20 targets of GPCR siRNA library score ranked according to z-score. The z-score for each target was calculated and targets were then ranked from lowest z-score up. The z-score of the top 20 targets ranged from -1.01202 to -2.35619.

5.3.4 Effect of plate of origin and location on the plate on identification of positive siRNA gene targets

Systematic bias can be introduced to high throughput screens as a result of mechanistic error associated with liquid handling robots. Bias resulting from location on plate or the individual plate that a target is located on was investigated (Figure 5.6). A heat map was created that represents the number of siRNA gene targets from the top 50 ranked targets in each well of the 96 well plate layout. The positive siRNA gene targets that sensitised GOhTRT cells to DCA induced cell death are distributed throughout the 96 well. The darker the colour, the greater the number of positive targets originated from that well.

The Dharmacon siGENOME libraries are organised largely alphabetically. This system can result in the positioning of gene family members in adjacent wells. A certain amount of functional similarity would be expected between gene family members and as such, similar phenotypes resulting from inhibition of each of these families is not surprising. Due to the organisation of the library in an alphabetical manner, clusters of gene family members in a specific area are unavoidable. Both heat maps representing positive siRNA gene targets indicates a cluster of targets from wells D10, D11, E10 and E11. These wells include the gene targets OPN1SW and OPN1MW and demonstrate that a degree of clustering of positive targets reflects the original alphabetical organisation of the siRNA library.

An evaluation of the plate of origin for each of the top 50 positive targets illustrated that there was no bias towards an individual plate (Table 5.3). Plate 7 appears to contribute a lower number of positive targets than the other plates as only 2% of z-score top 50 targets and none of the positive targets according to cell viability rankings. This is a result of the fact that plate 7 only contains 36 siRNA molecules as opposed to 80 siRNAs on the other

plates. The siGENOME (Dharmacon) siRNA libraries are organised alphabetically. As previously mentioned, clustering of positive siRNA gene targets on a particular plate may reflect the organisation strategy as opposed to being a limitation of the screening protocol. The lists of the highest ranked positive hits using both analytical strategies were quite similar (Table 5.2).

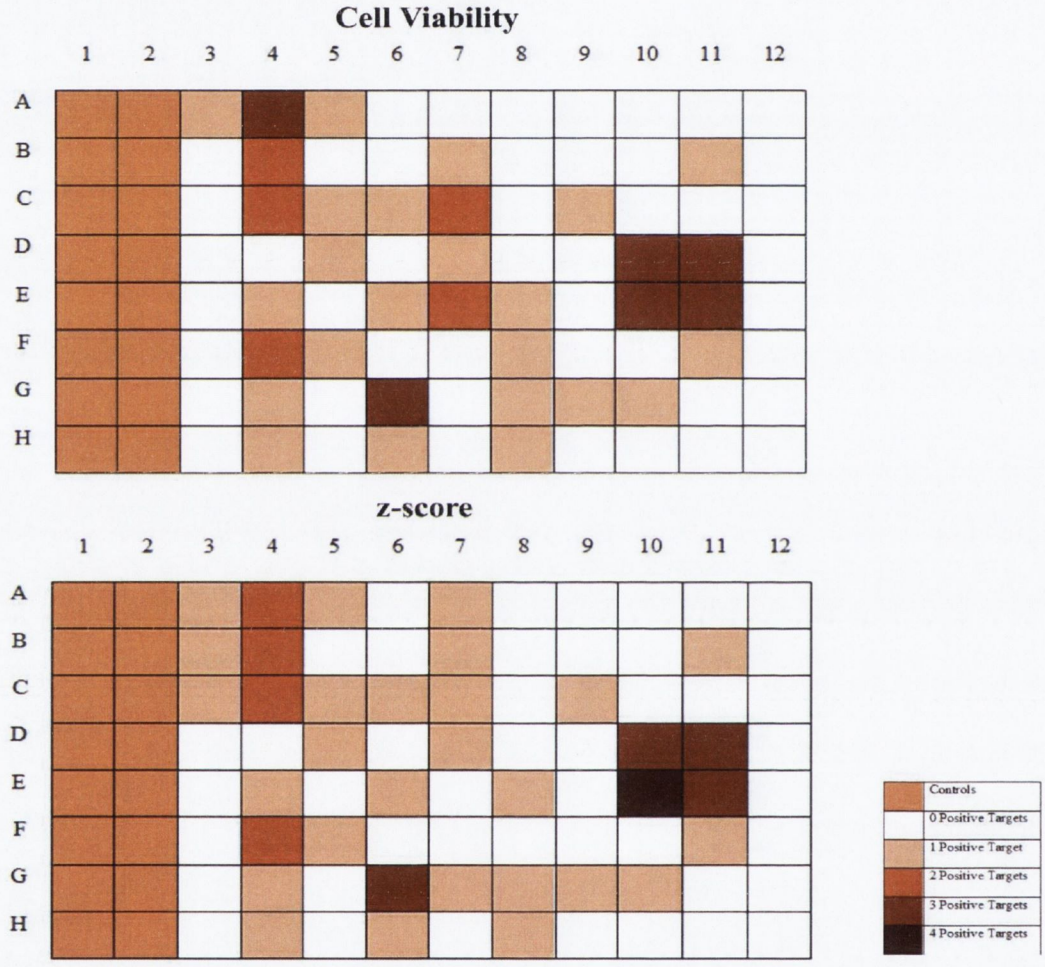


Figure 5.6: Distribution of positive targets across the plate layout. Heat maps describing the locations of the top 50 positive targets ranked by (A) cell viability and (B) z-score. Apparent clusters of positive targets in wells A3, A4, B3 and B4 are most likely resulting from the fact that the siRNA pools are arranged in the plates in alphabetical order of the targets. Targets that are members of the same family are located in sequence. It is not surprising that inhibition of members of the same family results in a similar phenotype.

There are 43 positive targets that were in the top 50 rankings of both analysis strategies. This would indicate that the strategy of ranking targets based on cell viability is a valid

strategy as it produces similar results to a previously published strategy using z-scores to rank the top positive targets (Turner *et al.*, 2008).

Table 5.3: Percentage of the top 50 ranked positive targets originating from each plate using both analytical methods - percentage cell viability and z-score.

	Cell Viability	z-score
Plate 1	2	2
Plate 2	20	18
Plate 3	16	10
Plate 4	16	24
Plate 5	24	20
Plate 6	22	22
Plate 7	0	4

5.3.5 Cellular processes linked with positive siRNA target genes

Ingenuity Pathway Analysis (IPA) software had previously been utilised to identify cellular pathways and processes that were connected to a cohort of DCA responsive genes involved in progression through the oesophageal carcinogenic sequence. This cohort of genes was identified using microarray gene expression analysis. The siRNA library screen carried out in this chapter has generated a cohort of genes that sensitise GOhTRT cells to DCA induced cell death. IPA was further utilised to assess the cellular pathways and processes that were linked to this new cohort of genes (Figure 5.7). The top 50 siRNA targets ranked by both cell viability and z-score were compared within the IPA software package.

The top processes characterised as 'Tox Lists' in IPA are thyroid receptor/retinoid X receptor (TR/RXR) activation, p53 signalling and retinoic acid receptor (RAR) activation. IPA facilitates a focused toxicity and safety assessment of compounds using toxicogenomics approaches. This function includes 'Tox lists' which are gene groupings based on critical processes and key toxicological response (IngenuitySystems, 2010).

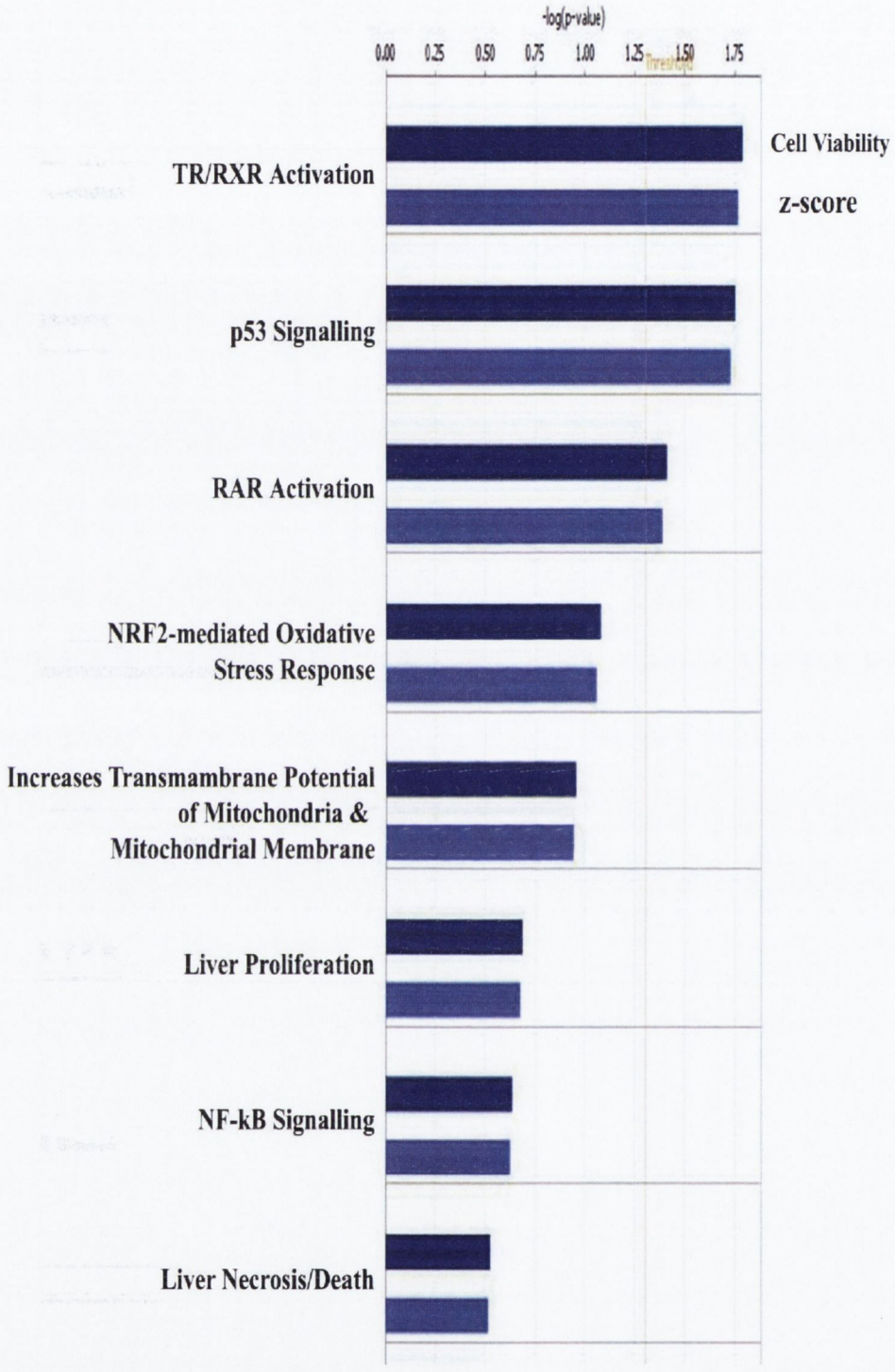


Figure 5.7: Functionality of modulators of sensitisation to DCA induced cell death in GOhTRT cells. IPA software was utilised to identify cellular processes that were associated with modulators of resistance to DCA induced cell death in GOhTRT cells. Signalling pathways identified that were statistical significant were TR/RXR activation, p53 signalling and RAR activation (p-value < 0.05, Fisher's Exact Test).

Phosphoinositide-3-kinase, catalytic, gamma and beta polypeptides (PIK3CG & PIK3CB) are two modulators of resistance to DCA induced cell death in GOhTRT cells that are involved in all three of these processes. These molecules are subunits of phosphoinositide-3-kinase (PI3K). PI3K interacts with a number of components of the p53 signalling pathway (Figure 5.8). These molecules include B-cell lymphoma 2 (BCL2), BCL2 like 1 (BCL2L1 aka BCL-XL), BCL2-associated X protein (BAX), cyclin-dependent kinase inhibitor 2A (CDKN2A aka p16), phosphatase and tensin homolog (PTEN), tumour protein p53 (TP53) and retinoblastoma 1 (RB1). The interaction of PI3K with these apoptosis related genes and the sensitisation of GOhTRTs to DCA induced cell death following inhibition of PI3K suggests a role for this signalling pathway in conferring resistance to DCA induced cell death. The connection with p53 signalling is important as cells with the loss of expression of p53 have been demonstrated to have selective advantage in the setting of OAC development (Maley, 2007)

IPA software also investigates cellular functions that are associated with gene lists. A selection of the top ranked cellular functions (p-value < 0.05; Fisher's exact test) associated with modulators of resistance to DCA induced cell death, proliferation, inflammation and DNA replication (Figure 5.9). Cellular functions and the specific genes from the cohort of modulators are summarised in Table 5.4. Canonical pathways associated with this cohort of genes were also identified (Figure 5.10). A number of interesting canonical pathways in the context of oesophageal adenocarcinoma development were identified including p53 signalling and NF-κB signalling. NF-κB and p53 have previously been linked with OAC and this study provides evidence for a role for DCA in OAC development.

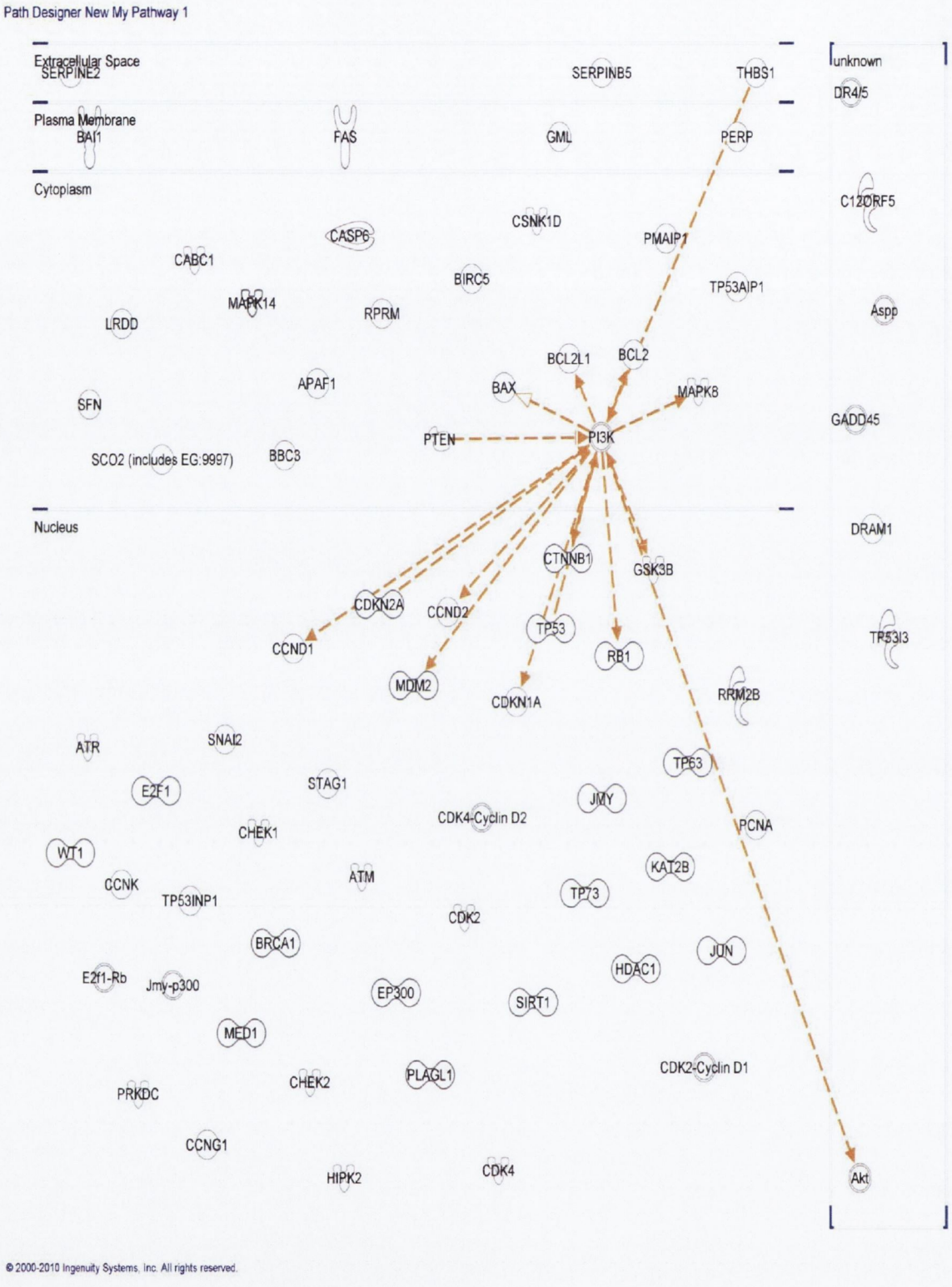


Figure 5.8: P53 signalling is linked to modulators of resistance to DCA in GOhTRT cells. The nodes from this network represent the p53 signalling pathway. IPA software connected the p53 signalling pathway with a cohort of modulators of resistance to DCA induced cell death in GOhTRT cells. PIK3CG and PIK3CB were central molecules that were involved in this pathway. Both of these molecules interact with a number of members of the p53 signalling pathway as indicated by the dashed lines.

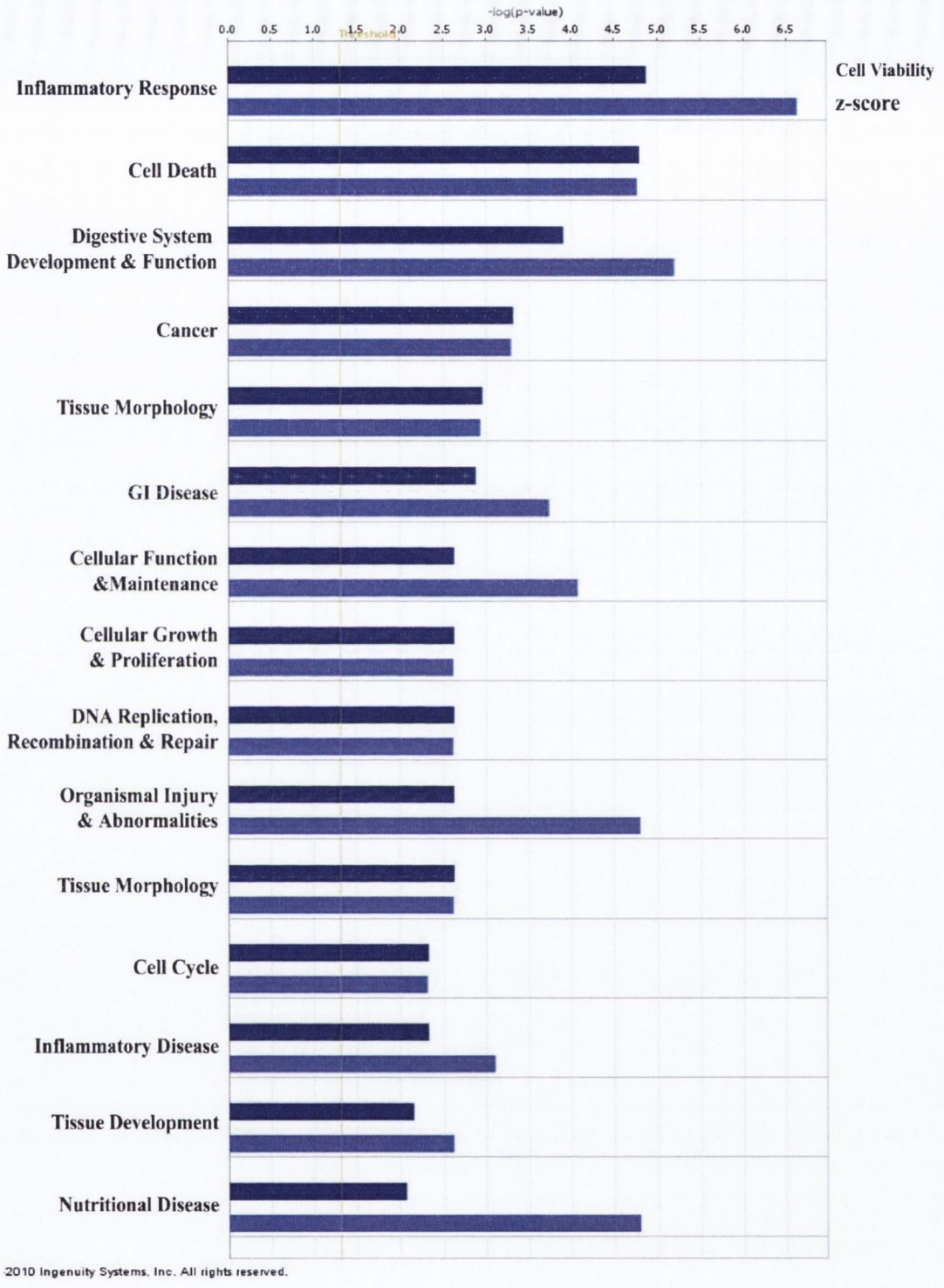


Figure 5.9: Cellular functions associated with modulators of resistance to DCA in GOhTRT cells. IPA software was utilised to investigate the cellular functions that are connected with a cohort of modulators of resistance to DCA induced cell death in GOhTRT cells. A selection of these cellular functions includes cell death, proliferation, inflammation and DNA replication. Cellular functions were scored and ranked using Fisher's Exact t-test. Resultant p-values < 0.05 were deemed statistically significant.

Table 5.4: Selection of cellular functions linked with modulators of resistance to DCA cell death in GOhTRT cells.

Category	Function Annotation	Molecules identified in screen	# Molecules
Cell Death	cell death of pancreatic cells	CXCR4, PIK3CG, TACR1	3
Cell Death	cell death of enteroendocrine cells	TACR1	1
Cell Death	apoptosis of pancreatic duct cells	CXCR4	1
Cell Death	apoptosis of pancreatic cells	CXCR4, PIK3CG	2
Cell Death	apoptosis of bone cell lines	PIK3CG, PTPN6	2
Cell Death	apoptosis of T lymphocytes	CCL25, CXCR4, PIK3CG	3
Cell Death	apoptosis of acinar gland cells	PIK3CG	1
Cell Death	survival of unspecified cell lines	PIK3CG	1
Cell Death	cell viability of cardiomyocytes	GLP1R	1
Cell Death	cell viability of nervous tissue cell lines	PTPN6	1
Cell Death	cell viability of cortical neurons	PTPN6	1
Cell Death	cell viability of normal cells	GLP1R, PTPN6	2
Cancer	transformation of embryonic cell lines	PIK3CB, PIK3CG	2
Cancer	transformation of cell lines	PIK3CB, PIK3CG, PTPN6, TACR1	4
Cancer	transformation of breast cancer cell lines	TACR1	1
Cancer	transformation of fibroblast cell lines	PIK3CB, PIK3CG, PTPN6	3
Cancer	hyperproliferation of adenocarcinoma	PIK3CG	1
Cancer	entrance of tumour cell lines	CXCR4	1
Cancer	infection of bone cancer cell lines	CXCR4	1
Cancer	endometrial adenocarcinoma	PTGFR	1
Cancer	adenocarcinoma	CXCR4, FZD3, PIK3CG, PTGFR	4
Cancer	metastasis of cancer cells	CXCR4	1
Cancer	metastasis of breast cancer cell lines	CXCR4	1
Cancer	acute myeloid leukaemia	CXCR4, HTR3A	2
Genetic Disorder	obesity	GLP1R, HTR7, HTR3A, PRLHR	4
Cellular Growth and Proliferation	proliferation of thyroid tumour cell lines	CXCR4	1
Cellular Growth and Proliferation	formation of endodermal cells	FZD1	1
Tumour Morphology	proliferation of adenocarcinoma	PIK3CG	1
Cell Cycle	arrest in G1/S phase transition of prostate cancer cell lines	CXCR1	1
Nutritional Disease	obesity	GLP1R, HTR7, HTR3A, PRLHR	4

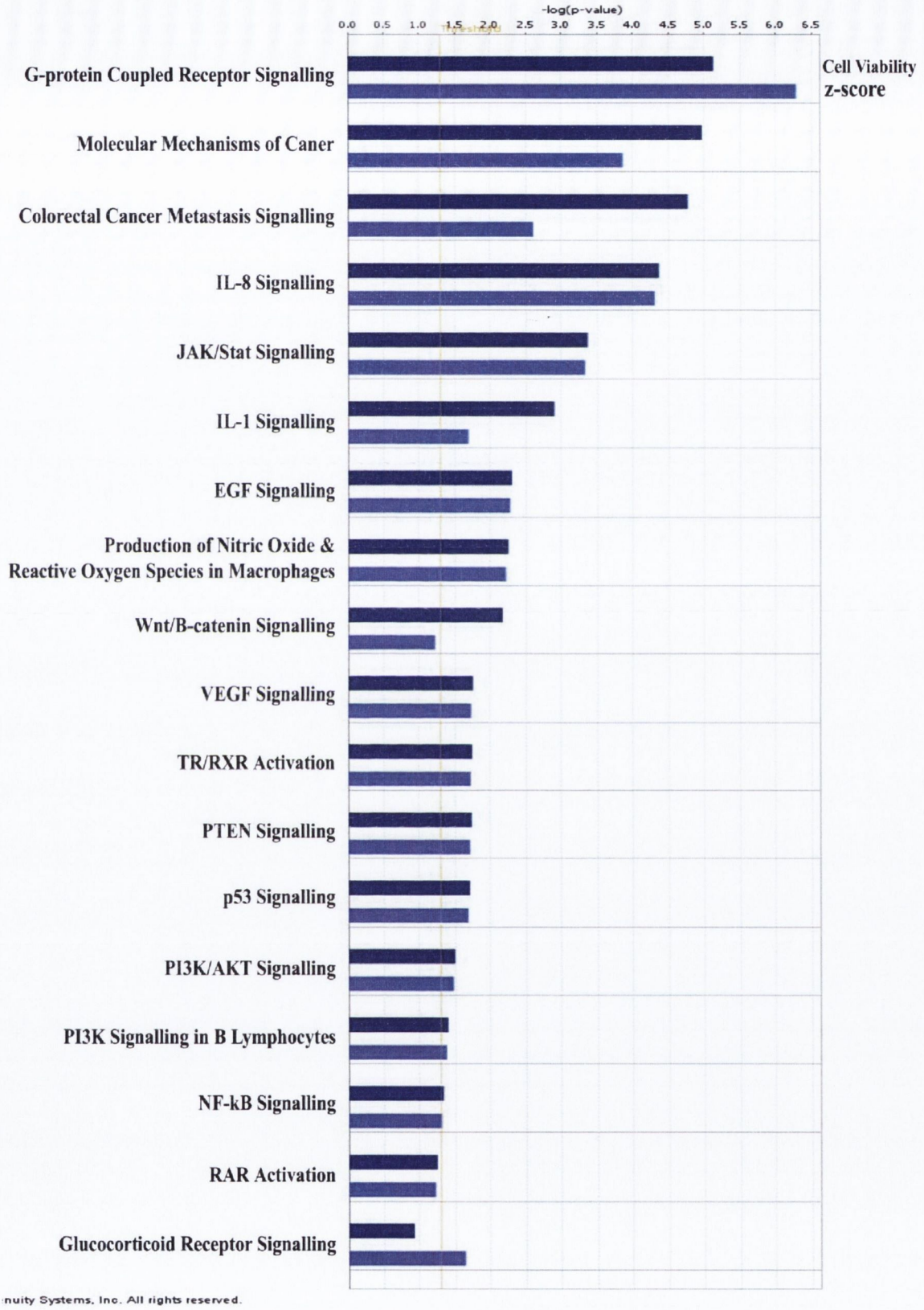


Figure 5.10: Canonical pathways associated with modulators of resistance to DCA induced cell death. IPA analysis was utilised to identify canonical pathways associated with modulators of resistance to DCA induced cell death in GOhTRT cells. A number of interesting pathways in the context of oesophageal malignancy were identified including p53 signalling and NF-κB signalling.

5.4 Discussion

An siRNA library screen to identify modulators of resistance to DCA induced cell death in oesophageal dysplastic cells was carried out (Figure 5.11). A library targeting GPCRs were chosen as these molecules represent 15% of the druggable genome and 25% of the current viable experimental and marketed drugs. The druggable genome is a set of genes coding for proteins that are capable of binding drug-like molecules. This cohort is comprised of approximately 30,000 different genes. The most interesting subset of the druggable genome is the genes that have been implicated in various diseases (Hopkins and Groom, 2002). This body of work aimed to sensitise an oesophageal dysplastic cell line to DCA induced cell death. DCA signalling in dysplastic cells is of particular interest as the extent of dysplasia correlates with risk of adenocarcinoma development (Buttar *et al.*, 2001) and preliminary experiments demonstrated that a dysplastic cell line was resistant to DCA induced cell death. A model system has been developed using an oesophageal dysplastic cell line, GOhTRT cells, that exhibits resistance to DCA induced cell death. Sensitisation of GOhTRT cells to DCA exposure following inhibition of each GPCR gene target in a library of 516 siGENOME (Dharmacon) siRNA pools was assessed using an MTT assay.

There are two methods that can be implemented in siRNA high throughput screens. One method involves the use of two or three individual siRNA molecules targeting the same gene in different wells. In order to reduce costs, this method frequently neglects to include replicates at the primary screen stage. The advantage of this screening strategy is that the number of positive targets can be selected based on a positive phenotype in two out of three wells or three out of three wells only.

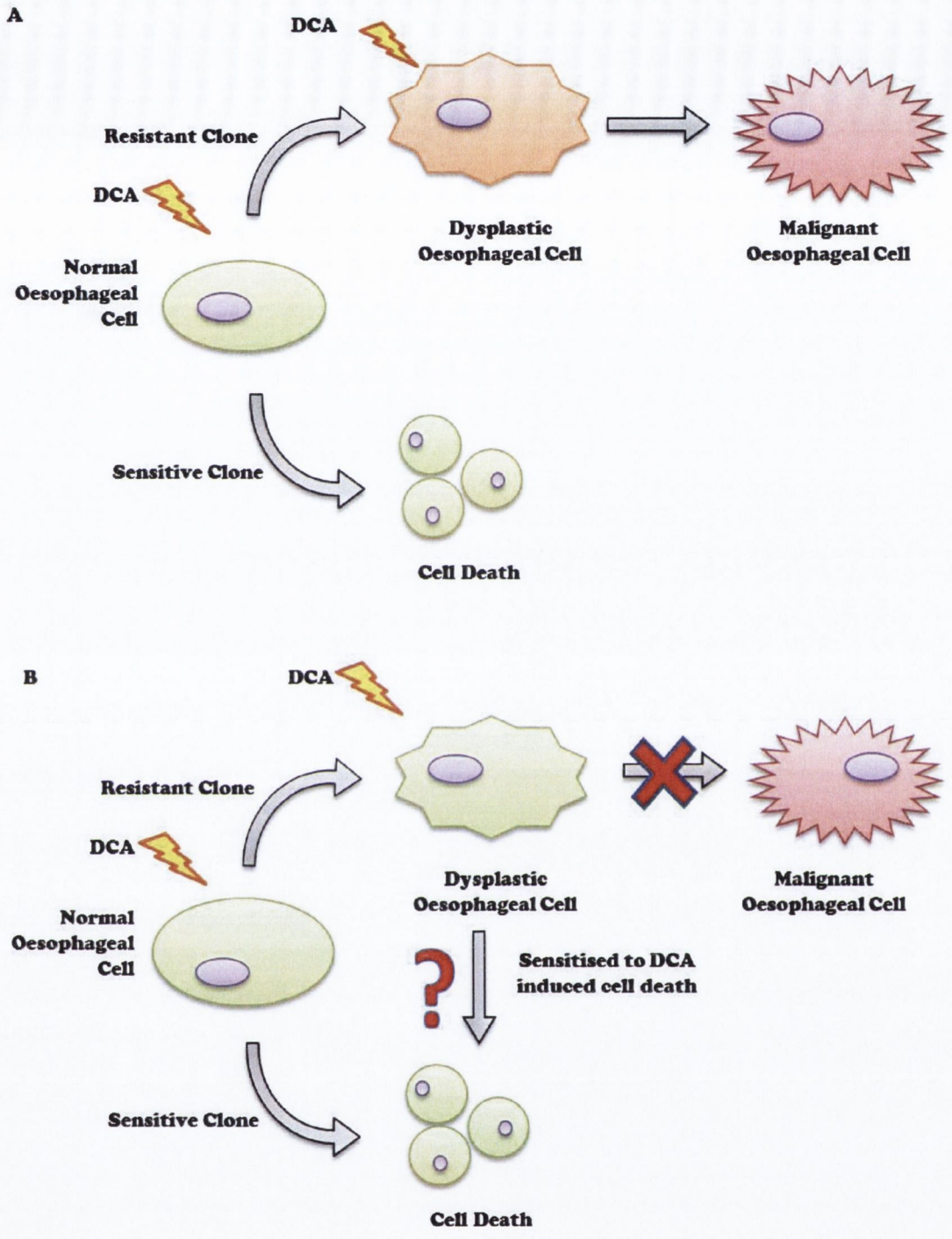


Figure 5.11: Schematic of the DCA response of normal and dysplastic oesophageal cells. (A) Normal oesophageal cells are generally sensitive to DCA induced cell death. The clonal expansion of mutated cells which display a more resistant phenotype to DCA induced cell death establishes an environment of genetic instability. This environment promotes the development of premalignant and malignant clones. (B) Sensitisation of the dysplastic cell to DCA induced cell death may present a viable target to develop a novel therapeutic strategy. This study aims to identify a target that would induce a cell death response to DCA in dysplastic cells using an siRNA library screen.

Using the second siRNA screening method involving pools of four non-overlapping siRNAs, it is impossible to determine in a primary screen how many siRNA molecules are successfully inhibiting the gene. However, a major advantage associated with pooled siRNAs is that the concentration of each siRNA is considerably lower than in the individual siRNAs but the final total concentration of siRNA per well remains the same. An advantage of having a lower concentration of the individual siRNAs in each well is the potential to reduce the number of off-target effects which in turn reduces the number of false positive results (Krausz, 2007). For these reasons, this study utilised siRNA pools targeting four non-overlapping siRNA molecules.

There were two approaches taken in the development of the analysis strategies utilised for this siRNA library screen. The first approach involved the determination of the percentage cell viability of each well following specific inhibition of one target per well by a pool of siRNA molecules. Cell viability was defined as the ratio of the absorbance values in the target well compared to the median of the negative controls expressed as a percentage. The second approach adopted was to rank the targets based on their z-score. The z-score is representative of the distance that a value is from the mean of the sample measured in standard deviations. Using the z-score to identify potential positive siRNA gene targets is a method that has been published in relation to siRNA screen analysis (Turner *et al.*, 2008, Azorsa *et al.*, 2009). It was possible to validate the relevance of the strategy adapted in this study of ranking siRNA target genes based on cell viability by comparing the lists derived by these two ranking methods. Comparison of both analysis strategies revealed a high level of correlation as 43 of the top 50 siRNA gene targets are shared by both strategies (Table 5.2). The position of each siRNA gene target in the top 50 ranked positive hits using both cell viability and z-score were extremely similar which would indicate consistency between the two analysis techniques.

A number of positive targets have been identified by both analysis methods which are potentially interesting in the context of oesophageal adenocarcinogenesis. These include interleukin 8 receptor alpha (IL8Rα), interleukin 8 (IL8), glucagon-like peptide 1 receptor (GLP1R) and frizzled homolog 1, 3 & 10 (FZD 1, FZD3, and FZD10).

IL8Rα is a high affinity receptor for the inflammatory related cytokine IL8. DCA has previously been demonstrated to induce expression of IL8 in oesophageal cells *in vitro* (Jenkins *et al.*, 2004). It has been shown in this siRNA library screen that independent inhibition of both IL8 (cell viability = 83%; p-value = 0.00069047; ranked number 63 by cell viability) and its receptor IL8Rα (cell viability = 76%; p-value = 7.07722E-05; ranked number 23 by cell viability) results in a sensitisation of GOhTRT cells to DCA induced cell death. This suggests a role for IL8 signalling in increasing the survival rate and possibly increasing proliferative potential of oesophageal dysplastic cells. IL8 has previously been implicated as a promoter of cell proliferation in human glioblastoma and human melanoma (de la Iglesia *et al.*, 2008, Gabellini *et al.*, 2009). The basal expression level of IL8 in an adenocarcinoma cell line (SKGT4) is 50 times greater than the basal level of IL8 in the Het-1A cell line (normal oesophageal squamous). IL8 expression is also inducible in response to DCA in both cell lines but to a greater extent in the SKGT4 cell line (Duggan *et al.*, 2010). Further investigations into the relationship between IL8 signalling and proliferation in oesophageal adenocarcinogenesis may reveal a link between the two and subsequently, IL8 and its receptor may be selected as novel therapeutic targets in treatment of oesophageal adenocarcinoma.

Inhibition of GLP1R sensitised GOhTRT cells to DCA induced cell death (cell viability = 63%; p-value = 0.000102773; ranked number 2 by cell viability). GLP1R is a promising candidate for *in vivo* targeting for diagnostic and therapeutic purposes. This receptor is

highly expressed in specific tumours but has low expression on normal tissue surrounding these tumours. A study investigating GLP1R expression has demonstrated that GLP1R is expressed in normal duodenum Brunner's glands and in the small and large intestine. GLP1R may present a novel target for *in vivo* scintigraphy and targeted radiotherapy (Korner *et al.*, 2007). GLP1R has also been demonstrated to inhibit H_2O_2 induced apoptosis in a mouse insulinoma cell line. It was determined that pre-treatment of these cells with GLP1R enhances the expression and/or activity of the anti-apoptotic proteins BCL2 and BCL-XL. Interestingly, PI3K appears central to this process (Hui *et al.*, 2003). If further investigations were to find an expression of this receptor on oesophageal tumour cells or even premalignant cells, GLP1R may also be followed up as a potential novel therapeutic target in the treatment of oesophageal carcinogenesis.

FZD 1, FZD 3 and FZD 10 inhibition also sensitised GOhTRT cells to DCA induced cell death (FZD1: cell viability = 81%, p-value = 0.013255268; FZD3: cell viability = 70%, p-value = 0.00619923; FZD10: cell viability = 69%, p-value = 0.000766204; ranked number 48, 9 & 8 by cell viability respectively). These frizzled homolog proteins are key components of the Wnt signalling pathway which has been implicated in oesophageal carcinogenesis (Clément *et al.*, 2007). Frizzled proteins act as receptors on the cell surface for Wnt proteins. Binding of Wnt proteins to members of the frizzled family initiates a signalling cascade that results in accumulation of β-catenin in the nucleus and transcription of β-catenin target genes. In oesophageal adenocarcinoma, Wnt2 is upregulated and adenomatous polyposis coli (APC), secreted frizzled-related proteins (SFRP) and Wnt inhibitory factor-1 (WIF1) (inhibitors of Wnt signalling) are downregulated by epigenetic methylation. This results in an accumulation of β-catenin in the nucleus with aberrant expression of target genes of the Wnt signalling pathway. It has been postulated that the Wnt signalling pathway could be a potential therapeutic target for the treatment of

oesophageal carcinogenesis. An antibody to the frizzled proteins is one therapeutic option that has been suggested (Clément *et al.*, 2007). This study suggests that blocking the Wnt signalling pathway by the targeted inhibition of FZD 1, FZD3 and FZD10 sensitises cells to DCA induced cell death. This indicates a further role for the Wnt signalling pathway and its downstream targets in oesophageal carcinogenesis by conferring a resistance to DCA induced cell death.

Inhibition of G-protein coupled receptor 23 (GPR23) results in a reduction in cell viability of GOhTRT cells to 63% (p-value = 5.03001E-05; Student's t-test; ranked number 3 by cell viability). GPR23 has been demonstrated to interact directly with B-cell lymphoma 6 (BCL6) via a protein-protein interaction (Miles *et al.*, 2005). BCL6 has been associated with increased apoptosis and delay of the cell cycle in an osteosarcoma cell line (Albagli *et al.*, 1999). Sensitisation of GOhTRT cells to DCA induced death following inhibition of GPR23 may indicate a novel role for GPR23-BCL6 protein interaction in regulation of cell death in oesophageal dysplasia. Further validation and investigation are required to confirm this novel function of this protein.

Chemokine (C-C motif) ligand 25 (CCL25) inhibition sensitises GOhTRT cells to DCA induced cell death (cell viability - 75%; p-value = 0.00024109, Student's t-test; ranked number 18 by cell viability). CCL25 increases activation of PI3K complexes in memory T cells, B cells and immature thymocytes (Ward, 2004). PI3K signalling has been linked with resistance to DCA induced cell death in GOhTRT cells through a number of avenues. As such, this potential novel function of PI3K requires further validation and investigation.

The positive siRNA gene targets for sensitisation of GOhTRT cells to DCA induced cell death described here are a sample of the top positive targets resulting from this primary

screen. The next step in the screening process would be a secondary validation screen targeting the top positive targets to confirm that specific inhibition of the intended gene, not off-target effects result in a sensitisation to DCA induced cell death. This screen was completed using Dharmacon siRNA pools contain 4 non-overlapping siRNAs targeting one gene. An appropriate validation method for this type of screen is to carry out the siRNA targeted inhibition using dissected siRNAs pools i.e. the pool of four non-targeting siRNA molecules separated into one individual siRNA molecule per well. A selection criteria can be predefined where only siRNA targets that are positive using at least 2 or more individual siRNAs are brought forward for future investigations (Whitehurst *et al.*, 2007). Alternatively, a range of non-overlapping siRNA molecules obtained from a different supplier could be used. This technique is useful to confirm that the phenotype characterised in the primary screen was valid and not a result of off-targets effects or supplier specific modifications. The siRNA libraries utilised in this screen are comprised of siGENOME siRNA molecules (Dharmacon). Dharmacon have subsequently developed siRNA molecules with a backbone modification that results in a decrease in off-target effects. These siRNAs are known as On-Target Plus siRNA (OTP) molecules. Non-overlapping OTP siRNA offers a validation strategy for use in secondary screens.

Subsequent validation screens which implement rescue experiments add considerable confidence to selected positive targets. These types of experiments can be somewhat technical but can add substantial weight to findings from the primary screen. These rescue experiments require the level of protein to be rescued or reverted to functional baseline level with continued siRNA inhibition of the target mRNA. There are a number of methods that can be utilised to achieve this rescue of protein expression. The transfection of an orthologous gene from a closely related species that could produce a functional protein without being inhibited by the siRNA should rescue the phenotype (Krausz, 2007).

Alternatively, using an siRNA that targets the 3' untranslated region (UTR) of the gene in conjunction with the transfection of construct of the target gene lacking the 3' UTR should result in a neutralisation of the effect of inhibition of the siRNA (Echeverri and Perrimon, 2006). A silent single point mutation in the target region of the gene of interest should also result in a cell resistant to RNAi for that gene. Kittler *et al* (2005) highlight a number of problems associated with the transfection of cDNA clones into cells. These problems include a lack of availability of clones corresponding to all genes that may be investigated. The experimental procedures involved are also expensive and time-consuming. Constructs from different species may not have equivalent promoter regions as the endogenous gene and as such may not rescue the expression of the gene that is inhibited. Overall, Kittler *et al* (2005) did not believe that an approach using a cDNA clone is applicable or reliable and instead proposed the use of a bacterial artificial chromosome (BAC). The BAC would carry the gene of interest from a closely related species along with 20kb of the upstream sequence. The selection of an siRNA molecule that is not likely to cross-react with the orthologous gene is of extreme importance. An siRNAs targeting the 3' UTR of the endogenous gene would be less likely to target the exogenous gene as similarity between species in this region is less common than in the coding region. This study used this technique to successfully confirm RNAi inhibition of a gene by rescuing expression using a BAC (Kittler *et al.*, 2005). An extension of this approach is a system where both the endogenous human gene and the exogenous orthologous gene are inhibited independently and simultaneously. The independent inhibition of each gene in separate wells should result in no alteration of phenotype whereas the simultaneous inhibition of both genes in one well would result in an altered phenotype (Krausz, 2007).

The number of targets under investigation in secondary and subsequent screens is generally much less than in the primary screens which allows for an increased complexity of assay

during validation screens. The primary screen in this study involved a high throughput approach using a cell proliferation assay to characterise the resulting phenotype. This assay involved only one parameter which facilitated the determination of cell viability and was well suited to the high throughput system in place in this study. High content analysis (HCA) utilises technological advances in automated fluorescent microscopy to examine multiple parameters simultaneously. Experimental studies incorporating HCA have the potential for gaining a greater insight into the phenotypic changes occurring following siRNA inhibition. In the case of this study, a validation screen featuring HCA investigation of known features of apoptosis would contribute a greater understanding than the one parameter cell proliferation assay which only demonstrates an increase or decrease in cell viability. The identification of a decrease in cell viability following siRNA inhibition may be a result of a decrease in proliferation or a complete halt in cell growth. Greater cell numbers in a negative control well would give a higher reading than cells in treated wells which have stopped growing as opposed to being lost due to apoptosis. While a decrease in cell proliferation rates following exposure to an siRNA is extremely important and interesting, this study aims to assess a sensitisation to cell death. A HCA assay targeting known features of apoptosis including nuclear condensation, alterations in mitochondrial potential and actin content would give a much greater insight into the functionality of the target siRNA molecules. An investigation utilising immunofluorescent techniques to detect the protein expression of activated caspases involved in cell death would also give a greater understanding into the underlying mechanisms of this process e.g. caspase-3 or caspase-9 identification. Alternatively, the reduction in cell viability may be a result of arrest in a particular phase of the cell cycle. Subsequent screens and validation experiments would confirm if cell death or cell cycle arrest is responsible for the reduction in cell viability.

The final validation of top positive targets from this screen requires an accurate assessment of the levels of mRNA and protein of the target of interest, following specific inhibition by siRNA. The mRNA levels will be assessed using real time RT-PCR. Western blotting and immunofluorescence will be used to assess protein levels. An in-depth functional analysis of the gene of interest should also be completed to confirm that the functionality of the gene is effectively reduced. These assays would be target specific. An example of the type of assay suitable for this purpose would be to determine the expression levels of a downstream target of the gene that is inhibited. For example, if the frizzled genes from this primary screen were validated in subsequent screens, a final test to determine if a sufficient reduction in the levels of functional protein is achieved would be to assess the activity of the Wnt signalling pathway following inhibition of these frizzled genes.

This screen is a primary screen and the main function of such screens is to identify potential positive targets in a high throughput fashion. Validation screens, functional analysis and numerous other investigations are required to confirm and identify true potential therapeutic targets. The semi-automated siRNA transfection protocol and the assay developed here are powerful tools in the initial identification and can easily be adapted as the primary step in many other disease models. There are many combinations of cell lines and assay that may be integrated into this system to develop a panel of potential novel therapeutic targets.

A systems biology approach was implemented using Ingenuity Pathways Analysis (IPA) software to investigate the functional significance of modulators of resistance to DCA induced cell death in GOhTRT cells. IPA software categorises pathways and processes in a number of different ways to simplify interpretation of results. The three categories that were most interesting in the context of oesophageal malignancies were 'Tox lists', cellular

functions and canonical pathways. A number of pathways and processes appear in more than one category, possibly highlighting their significance.

'Tox lists' are gene groupings based on critical processes and key toxicological response. There were three statistically significant (p-value < 0.05, Fisher's Exact t-test) processes identified in a comparison analysis of the top 50 siRNA gene targets ranked by cell viability and z-score. These processes were thyroid receptor/retinoid X receptor (TR/RXR) activation, p53 signalling and retinoic acid receptor (RAR) activation. TR/RXR activation involves the formation of a TR/RXR heterodimer. This complex, in conjunction with a number of co-activators is a regulator of gene expression. TR/RXR also activated the phosphoinositide-3-kinase (PI3K) pathway. Previous work involving a chemical inhibitor screen demonstrated that inhibition of PI3K using the chemical inhibitor wortmannin sensitised GOhTRT cells to DCA induced cell death (Figure 4.8H). TR/RXR is involved in a number of biological processes such as growth, development and metabolism.

Loss of p53 functionality has previously been associated with oesophageal adenocarcinoma (Jenkins *et al.*, 2002, Maley, 2007, McCabe and Dlamini, 2005). Two modulators of resistance to DCA induced cell death, PI3K subunits PIK3CG and PIK3CB have been associated with p53 signalling pathway. PI3K interacts with a number of components of the p53 signalling pathway including the apoptosis associated proteins BCL2, BCL-XL and BAX. Altered expression of these proteins has previously been associated with progression through the oesophageal adenocarcinoma sequence (van der Woude *et al.*, 2002). Targeting of the PI3K/Akt/mTOR pathway has been proposed to overcome therapeutic resistance in acute myelogenous leukaemia (AML). Small molecule inhibitors have been tested *in vivo* and *in vitro* to target the PI3K/Akt/mTOR as in the case

of AML, activation of this pathway confers therapeutic resistance (Martelli *et al.*, 2007). The siRNA library screen carried out in this body of work has identified two components involved in PI3K signalling as modulators of resistance to DCA induced cell death in GOhTRT cells. Further investigation and validation may confirm the role of the PI3K pathway in conferring resistance to cell death in oesophageal dysplastic cells. Novel therapeutic strategies could be developed to target the PI3K pathway alone or in combination with other oesophageal treatment.

Unsurprisingly, functions such as cell death and cell proliferation are associated with the cohort of genes identified by this siRNA library screen. More specifically, modulators of resistance to DCA induced cell death have been linked with all facets of cell health including apoptosis, cell viability and cell survival (Table 5.4). Of interest, cancer related functions that were identified include the transformation of breast cancer cell lines and fibroblasts and also the hyperproliferation of adenocarcinoma. These findings link modulators of resistance to DCA induced cell death with transformation of cancer cell lines and hyperproliferation of adenocarcinoma. Again, the PI3K subunits are central to these functions. Investigation into the canonical pathways associated with modulators of resistance to DCA induced cell death have identified molecular mechanisms of cancer as one of the most statistically significant canonical pathways (p-value < 0.05; Fisher's Exact t-test). This provides further evidence linking modulators of DCA resistance with carcinogenesis. This is important as these data indicate that DCA may act as a carcinogen in oesophageal adenocarcinogenesis.

The systems biology analysis utilising IPA software has identified processes and molecules that have previously been linked with oesophageal adenocarcinogenesis and other malignancies. This validates the siRNA screening protocol that was implemented in this

study. The investigation into the functionality of modulators of DCA resistance has also linked a number of these modulators with the development and progression of cancer. As such, these processes and modulators of resistance to DCA induced cell death are promising candidates for novel therapeutic strategies in the treatment of oesophageal adenocarcinogenesis.

This study has demonstrated that DCA reduces the cell viability of oesophageal cell lines, with the cell lines representing the early stages of the carcinogenic sequence being more sensitive to DCA induced cell death. However, cell lines representing end stages of the oesophageal carcinogenic sequence are resistant to DCA induced cell death. Resistance to DCA induced cell death creates a genetically unstable environment and can select for cells that have acquired traits associated with malignancy. The development of malignancy in DCA resistant cells is most likely due to the introduction of double strand breaks in DNA resulting from DCA exposure (Jolly *et al.*, 2004). Resistant cells have acquired a trait which allows survival following DCA exposure which results in prolonged exposure to the bile acid and the development of further molecular irregularities. Dysplastic cells with an acquired resistance to DCA are the perfect environment for the development of genetic instability and further progression towards adenocarcinoma development. The presence of dysplasia has been demonstrated to increase the risk of adenocarcinoma development (Buttar *et al.*, 2001). The contribution of bile acids to the development of oesophageal adenocarcinoma has been well documented. Evaluation of bile concentrations present in patients with various stages oesophageal disease has established that each defined stage of oesophageal disease has a distinct pattern of concentrations of bile acids and also the type of bile acids that are present. There were increased levels of secondary bile acids such as deoxycholic and taurodeoxycholic acids detected in patients with erosive oesophagitis and BO (Nehra *et al.*, 1999). Barrett's oesophagus patients have a higher level of bile

associated reflux when compared to oesophagitis patients and normal control groups (Debruyne *et al.*, 2001). Studies such as this, that identify modulators of resistance to DCA induced cell death, are extremely important in the development of novel therapeutic strategies to prevent oesophageal adenocarcinoma.

Bile acids are clearly an integral element in the development of oesophageal adenocarcinoma. It has been demonstrated in this chapter that BCL-XL, IL8Rα, FZD 1, 3 & 10 and GLP1R are members of a cohort of identified modulators of resistance to DCA induced cell death. A selection of this cohort needs to be validated in secondary siRNA screens. A number of processes have been identified using a systems biology approach including p53 signalling and RAR activation. These processes represent positive avenues for future investigations and also the future development of anti-cancer therapies.

5.4.1 Technical discussion

Following completion of the siRNA screen targeting a library of GPCR molecules, quality assurance and quality control (QA/QC) was carried out on all the replicates of all plates. During the course of the experiment, a small number of wells were identified that were contaminated and were removed from the study. The siRNA gene targets that were affected were taste receptor, type 2, member 39 (TAS2R39), frizzled homolog 2 (FZD2), regulator of G-protein signalling 16 (RGS16) and dopamine receptor 1 (DRD1). Grubb's test for outliers was used to determine if there were any outliers. Scatterplot and boxplot analysis highlighted a systematic bias in the first replicate of plates 1 and 6. It was decided to remove replicate 1 from each of the 7 plates. This bias may be a result of the cleaning protocol that was used between each set of four replicates. Residual detergent in the pipettes may have interfered with siRNA transfection efficiency or directly altered cell viability. As each plate was replicated four times, loss of one replicate out of four still

allowed the target to be included in the final analysis. The remaining three values maintain statistical power. If two or more values for siRNAs targeting were lost, these targets were excluded from the final analysis.

It was demonstrated that there was no bias towards any one plate using either analytical method (Table 5.3). The plates of origin of the top 50 ranked positive hits appear to be randomly dispersed and the location of the target does not appear to influence ranking. Plate 7 does contribute less positive hits than the other plates but this is due to the fact that this plate only contains 36 targets as opposed to 80 targets on the other plates. Of note, the majority of siRNA targets are organised in the plates alphabetically. This should be considered as a contributing factor to the clustering of positive siRNA target genes on one plate as this organisation strategy results in families of siRNA targets being located together. The location of the top 50 positive targets was dispersed throughout the plate.

There were a small number of plate locations that were identified during the assessment of the liquid handling robots that may contribute to false positives. Positive targets ranked by either z-score or cell viability were examined to investigate if they originated from these areas. The main area of concern was identified as row H wells 9-12 during the assessment of the accuracy and consistency of the Matrix Hydra II™ liquid handling robot (Figure 4.2). There was no evidence to indicate that there were false positives originating from these areas. In fact, there were no positive targets identified from these wells in the top 50 positive targets. Future validation studies will encompass a more extensive list of top targets and positive targets may originate from this area. Additionally, the concentration of siRNA used in each well was in excess of what was required to inhibit expression. This excess of siRNA allows for a less stringent requirement on the levels of consistency within the needles.

Chapter 6

Results

The Investigation of CDX2 Homeostasis and Inducibility in Oesophageal Cell Lines Through the Development of a High Throughput siRNA Library Screening Strategy

6 CDX2 homeostasis and inducibility in oesophageal cells

6.1 Introduction

CDX2 is a transcription factor involved in the axial patterning of the alimentary tract during embryonic development and the maintenance of adult intestinal epithelial homeostasis. Target genes of CDX2 include mucin, sucrose isomaltase, villin 1, defensin 5 and alkaline phosphatase which are involved in cellular functions such as cell differentiation, cell proliferation, adhesion and apoptosis (Vaninetti *et al.*, 2009).

Oesophageal tissue is normally negative for CDX2 expression (Groisman *et al.*, 2004), however CDX2 induction in oesophageal cells appears to be an early event in the development of Barrett's metaplasia (Groisman *et al.*, 2004, Hu *et al.*, 2007). The expression of CDX2 increases following transition through the oesophageal carcinogenic sequence from normal to cardiac mucosa to Barrett's metaplasia (Vallbohmer *et al.*, 2006). This level of expression declines with the development of high grade dysplasia and oesophageal adenocarcinoma (OAC) (Vaninetti *et al.*, 2009, Phillips *et al.*, 2003). Research into colon carcinogenesis has indicated a potential role for CDX2 as a tumour suppressor gene (Bonhomme *et al.*, 2003, Guo *et al.*, 2004, Kim *et al.*, 2004). Hence, while CDX2 may be an important gene in the development of intestinal type metaplasia of the oesophagus, its anti-oncogenic properties may play a role in the prevention the development of OAC.

Bile salts have been implicated as contributors to the development of oesophageal tissue injury (Kazumori *et al.*, 2006) through a range of possible mechanisms including DNA damage (Zhang et al., 2009a) and induction of inflammation (Jenkins *et al.*, 2002). A number of DCA responsive genes have also been shown to be of importance in progression through the oesophageal carcinogenic sequence including genes involved in proliferation,

cell death and cellular movement (Duggan *et al.*, 2010). Some reports in explant systems have indicated that bile salts and acid have the capacity to induce CDX2 expression in oesophageal cells derived from GORD patients with BO in an NF-κB dependent manner (Huo *et al.*, 2010). These data demonstrated that exposure over a prolonged period of time to a cocktail of bile salts in a pulsatile fashion results in the induction of expression of CDX2 in oesophageal squamous mucosa.

NF-κB has been identified as a key transcriptional regulator of CDX2 expression (Huo *et al.*, 2010, Kazumori *et al.*, 2006). The p50 and p65 subunits of NF-κB have been demonstrated to have opposing effects on CDX2 expression with the p50/p50 homodimer stimulating CDX2 expression and p65/p50 heterodimer inhibiting CDX2 expression (Kim *et al.*, 2002). NF-κB expression increases steadily from little or no expression in normal squamous oesophageal epithelium, through to oesophageal OAC where maximal expression levels are observed (Abdel-Latif *et al.*, 2009).

Development of oesophageal OAC from premalignant BO appears to be associated with a reduction in CDX2 expression (Guo *et al.*, 2004, Vaninetti *et al.*, 2009). Expression levels of CDX2 in BO are markedly increased in comparison to normal tissue, and then decreased from BO to OAC development (Phillips *et al.*, 2003). A shift in cell signalling may occur in BO cells which allows for a suppression of CDX2 expression which further facilitates subsequent OAC development. As illustrated in the previous paragraph, NF-κB subunits p50 and p65 have opposing effects on CDX2 transcriptional regulation (Kim *et al.*, 2002). This may indicate a regulatory switch in NF-κB transcriptional control of CDX2 as an important event in progression from BO to OAC. CDX2 transcription control appears to be an extremely important process in progression through the oesophageal carcinogenic sequence. Elucidating the upstream cell signalling pathways involved in the response of

CDX2 to bile salts and acid could be of extreme importance in the development of therapeutic strategies targeting prevention of progression from BO to OAC.

6.2 Objectives

High throughput siRNA screening is a powerful tool in the identification of signalling pathways involved in a cellular response to external stimuli. Bile salt signalling is suggested to play a role in the control of CDX2 expression. The main aim of this chapter was to develop an siRNA screening assay to be integrated into a semi-automated protocol to determine the upstream signalling pathways involved in CDX2 transcription regulation in response to bile salts. The assay development required:

- the establishment of the basal levels of CDX2 expression in a number of cell lines.
- the assessment of the transcriptional response of CDX2 to bile salts and acid.
- the identification of potential positive controls for the semi-automated siRNA library screening transfection protocol.
- the optimisation of the siRNA transfection conditions.

6.3 Results

6.3.1 CDX2 basal mRNA expression levels in cell lines originating from the gastrointestinal tract

The basal CDX2 mRNA levels of a number of cell lines originating from the gastrointestinal tract were determined using real-time RT-PCR. CDX2 expression is absent in normal oesophageal tissue (Phillips *et al.*, 2003) and also in the normal squamous oesophageal cell line, Het-1A (Hu *et al.*, 2007). As such, Het-1A cells were used as the calibrator to determine the levels of CDX2 in the other cell lines: AGS (gastric OAC); GOhTRT (oesophageal dysplastic); OE33 (oesophageal OAC - poorly differentiated);

HCT116 (colorectal carcinoma); Caco2 (colorectal adenocarcinoma). CDX2 mRNA expression levels were calculated using the delta-delta CT relative quantification method.

The highest level of CDX2 mRNA expression was observed in AGS cells with ~5 000 fold increase in expression of CDX2 compared to Het-1A cells. CDX2 mRNA expression in the other cell lines analysed, Caco2, HCT116 and OE33 exhibited ~700, ~550 and ~400 fold increase respectively compared to Het-1A cells. The oesophageal dysplastic cell line GOhTRT expresses CDX2 at a lower level than the Het-1A cells (~40%) (Figure 6.1A).

In order to establish if the observed expression of CDX2 resulted in a functionally active protein, the basal mRNA expression level of Villin 1 (VIL1), a downstream target of CDX2 was determined (Figure 6.1B). VIL1 expression levels were higher by a significant amount in all cell lines compared to Het-1A, with the exception of GOhTRTs. This would imply that the increased CDX2 expression levels in the AGS, OE33, HCT116 and Caco2 cell lines compared to Het-1A cells also resulted in an increase in functionally active CDX2 protein resulting in induction of the CDX2 target gene, VIL1.

6.3.2 CDX2 mRNA expression in Het-1A cells following exposure to a bile acid cocktail in neutral and acidified media

Increased CDX2 mRNA expression has been documented to occur in the development of BO from normal squamous tissue (Phillips *et al.*, 2003, Vallbohmer *et al.*, 2006). Exposure to DCA (1000μM; 8 hours) has been shown to induce CDX2 mRNA expression in Het-1A cells (Hu *et al.*, 2007). It has previously been shown that exposure of Het-1A cells to DCA at this concentration for 6 hours results in a reduction in cell viability to ~20%. A decrease in cell viability of Het-1A cells (~15%) in response to DCA (1000μM; 6 hours) was consistently observed in a chemical inhibitor screen where this concentration

of DCA was used as a positive control (Figure 4.8). Based on these data, it was decided to examine the ability of a bile salt cocktail at a lower concentration of 400μM in neutral or acidified medium to induce CDX2 mRNA expression in Het-1A cells using RT-PCR.

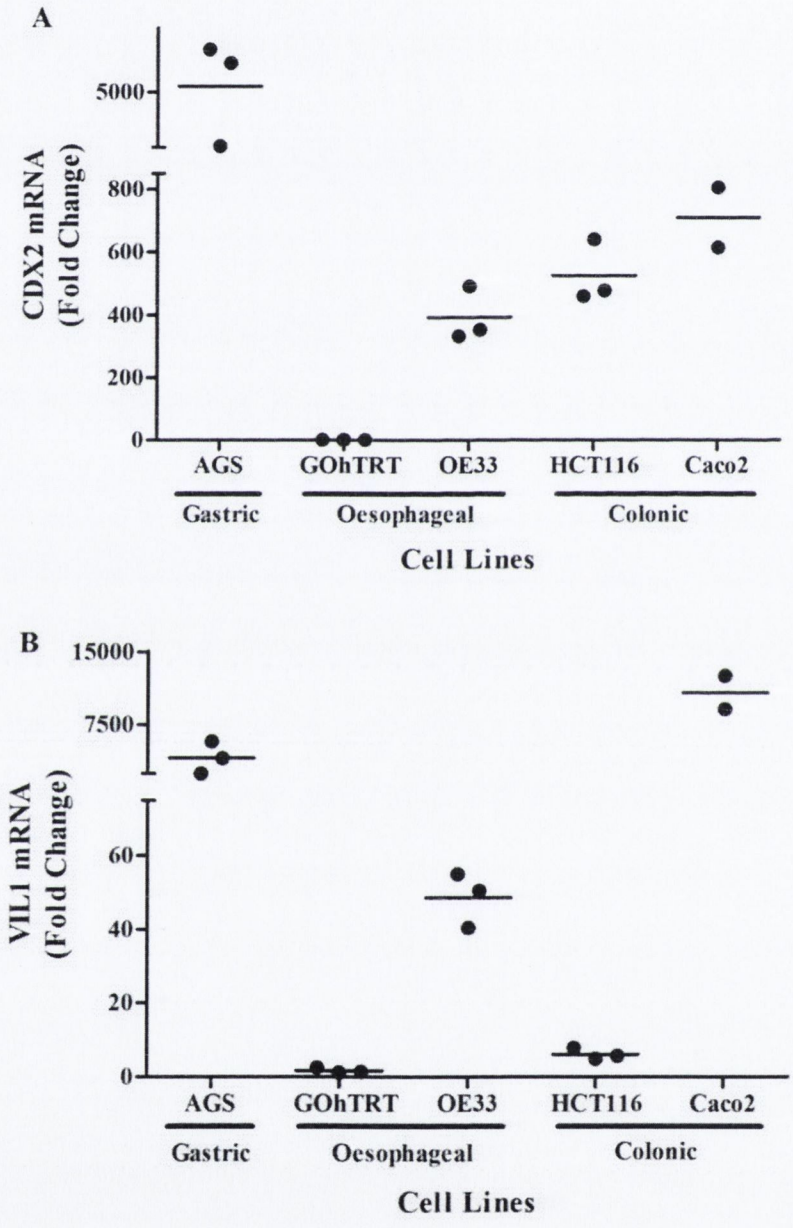


Figure 6.1: Basal levels of CDX2 mRNA transcription in the oesophageal OAC cell lines. The CDX2 mRNA expression level in OE33 cells is approximately 400 times greater than in Het-1A cells. The mRNA level of (A) CDX2 and (B) VIL was determined using real-time PCR. The fold change is representative of the difference in mRNA expression of each gene between Het-1A cells and each cell line.

Exposure of Het-1A (normal oesophageal squamous epithelium) cells to a bile salt cocktail [400μM] in neutral (pH7.4) and acidified (pH5.5) media for 3 days with 3 ten minute pulses daily resulted in an induction of CDX2 mRNA expression (Figure 6.2 A) . However, this response was inconsistent in the degree of induction observed (Figure 6.2 B) and was also completely absent in some replicates (Figure 6.2 C). The variation between technical replicates was frequently higher than is normally accepted using this technique. In one repeat of this experiment, CDX2 mRNA expression was undetermined across all treatments by the real-time PCR protocol that was implemented. These inconsistencies in the level of induction of CDX2 expression are due to the low copy number of CDX2 that is present in the normal oesophageal cells. CDX2 expression is induced by a bile acid cocktail however further investigation is required to confirm the levels of induction.

As previously described, the relative quantification analysis technique was used in these experiments. A low Ct value is indicative of a high initial copy number whereas a high Ct value indicates a low level of initial copy number of a gene. The Ct values of CDX2 following exposure to bile salts with and without acid were above 35 in all experiments for all treatments in this cell line. This indicates that the detection of CDX2 expression in this system is a very late event which would imply that the levels of CDX2 copy number in Het-1A samples, both resting and treated, were extremely low. This low level of CDX2 expression reduces the reproducibility of these results and makes it difficult to reach a definitive conclusion as to the level of induction of CDX2 in Het-1A cells in response to exposure to bile salts.

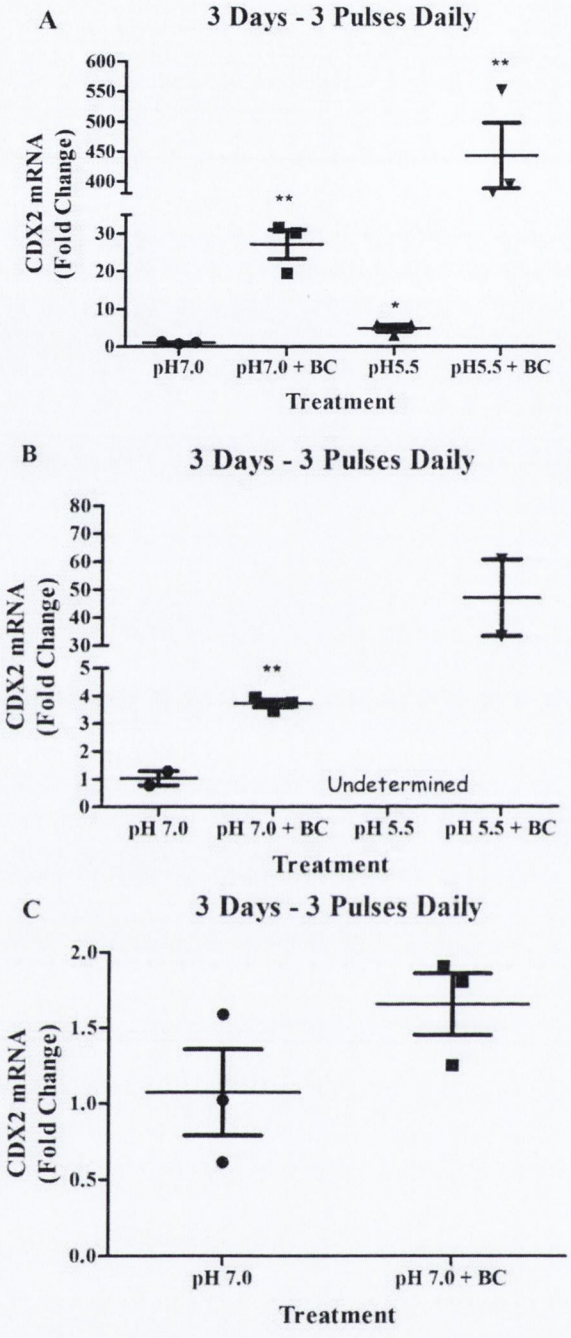


Figure 6.2: Induction of CDX2 mRNA expression in Het-1A cells following exposure to bile salts and acid was inconsistent and difficult to reproduce. Het-1A cells were exposed to a bile salt cocktail [400μM] in neutral (pH7.4) and acidified (pH5.5) media for 3 days with 3 ten minute pulses daily. (A) The first exposure of Het-1A cells resulted in a large increase in CDX2 expression following treatment with a bile salt cocktail in both neutral and acidified media. (B) This trend was repeated in the second exposure however the level of CDX2 induction had decreased and was completely lost following a (C) third exposure. A fourth exposure was carried out, however CDX2 mRNA expression levels were undetermined in these samples. *p-value < 0.05; **p-value < 0.01 (Student's t-test – compared to untreated control pH7.4; BC = Bile salt cocktail).

6.3.3 CDX2 & VIL1 expression levels in OE33 cells following exposure to DCA

CDX2 expression in response to DCA exposure in OE33 malignant cells was assessed using real-time RT-PCR and Western blotting. OE33 cells were exposed to DCA at a range of concentrations (0-300μM) for 8, 10 or 24 hours. CDX2 mRNA expression levels are decreased by statistically significant levels following exposure to DCA at concentrations of 100μM and 150μM at 8, 10 and 24 hours. Additionally, exposure to 50μM and 300μM DCA for 24 hours was also statistically significant, as was exposure for 8 hours of 300μM DCA (Figure 6.3). Western blotting was carried out using anti-CDX2 rabbit monoclonal antibody on nuclear protein fractions of OE33 cells that had been treated with DCA [150μM] over time. This western blotting demonstrated a reduction in CDX2 protein following 8 hours exposure to DCA [150μM] (Figure 6.4).

In order to establish whether the reduction in CDX2 was biologically significant, mRNA expression levels of VIL1, a downstream target gene of CDX2, were also determined. DCA exposure for 10 and 24 hours at concentrations of 25, 50, 100 and 150μM decreased VIL1 expression significantly, as did 8 hours of exposure to DCA at 25 and 150μM (Figure 6.3, Table 6.1). These findings indicate that the functionality of CDX2 is reduced to a biologically significant level in OE33 cells in response to DCA exposure.

6.3.4 Cell viability of OE33 cells following exposure to DCA

The viability of OE33 cells exposed to DCA [150μM] was assessed using an MTT cell proliferation assay as DCA has potentially toxic effects on cells. Cell viability of OE33s was not reduced by a significant level following exposure to DCA [150μM] for a total of 24 hours (Figure 6.5). Interestingly, cell viability was increased by a statistically significant level following exposure to DCA [150μM] for 7.5 hours. This time point correlates with a decrease in CDX2 activity to less than 50% of resting levels. CDX2 has

previously been shown to reduce cell growth in HT29 and Caco2 (Guo *et al.*, 2004) and these data suggest a similar role for CDX2 in OE33 cells. Further analysis using an siRNA to CDX2 in conjunction with a cell proliferation assay may confirm this hypothesis.

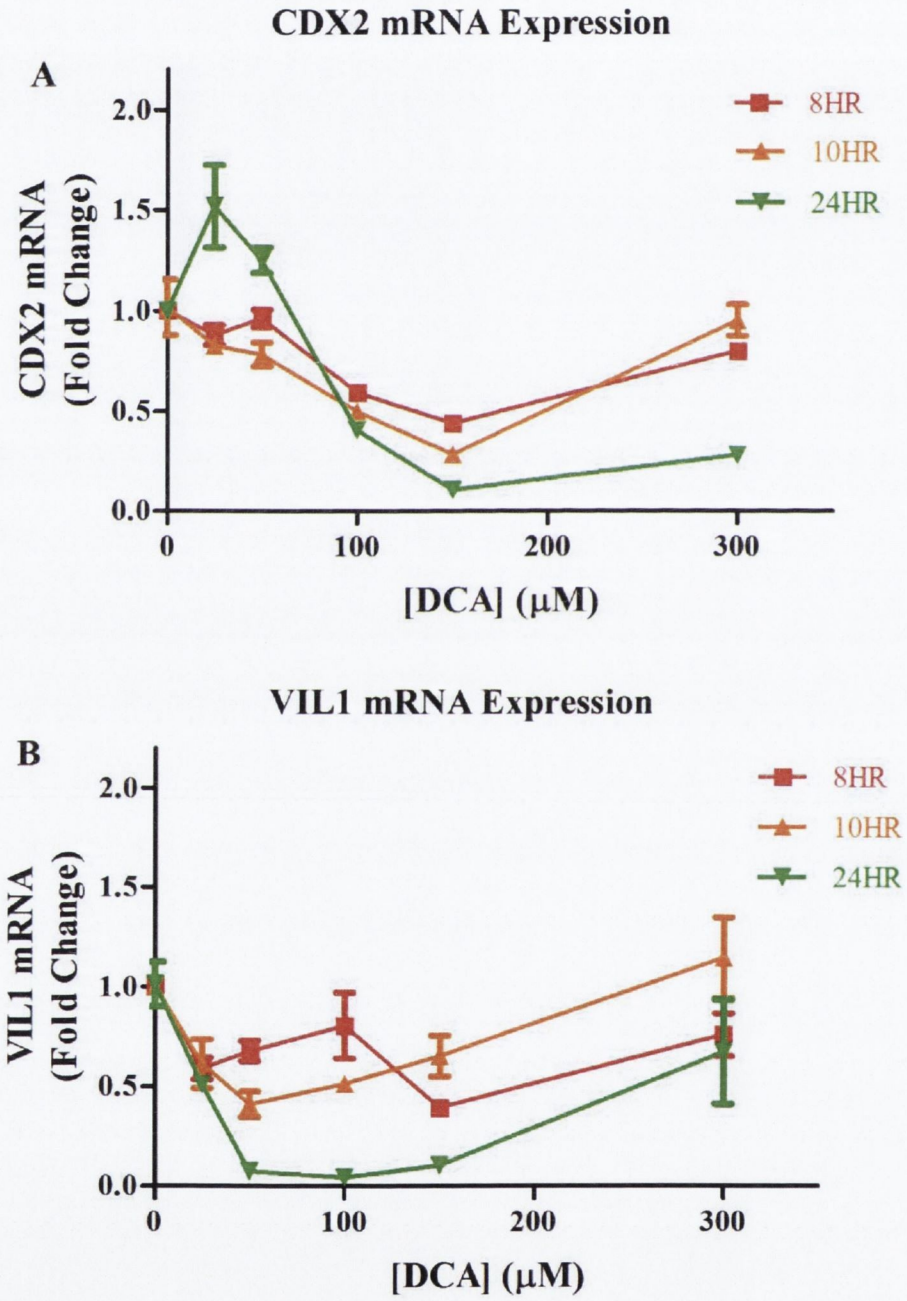


Figure 6.3: CDX2 and VIL1 mRNA expression in OE33 cells following exposure to DCA over time. (A) DCA exposure (100 and 150μM) for 8, 10 and 24 hours reduces CDX2 transcription to a statistically significant level (Table 6.1). (B) The biological relevance of this decrease in CDX2 is confirmed by the reduction of VIL1, a CDX2 target gene, mRNA expression at 8, 10 and 24 hours.

Table 6.1: P-values for CDX2 mRNA expression in OE33 cells exposed to DCA

	8 Hours		10 Hours		24 Hours	
[DCA]	***CDX2***	***VIL1***	***CDX2***	***VIL1***	***CDX2***	***VIL1***
25μM	0.124014	0.027509	0.249629	0.037395	0.064005	0.012834
50μM	0.551453	0.052833	0.193903	0.001218	0.016543	0.007424
100μM	0.001812	0.357724	0.020051	0.000364	0.000188	0.000981
150μM	0.000337	0.005208	0.006218	0.031967	1.07E-06	0.001346
300μM	0.007542	0.177099	0.706552	0.516859	3.14E-06	0.297664

p-values determined using Student's t-test; compared to untreated controls; significance set to <0.05 (red).

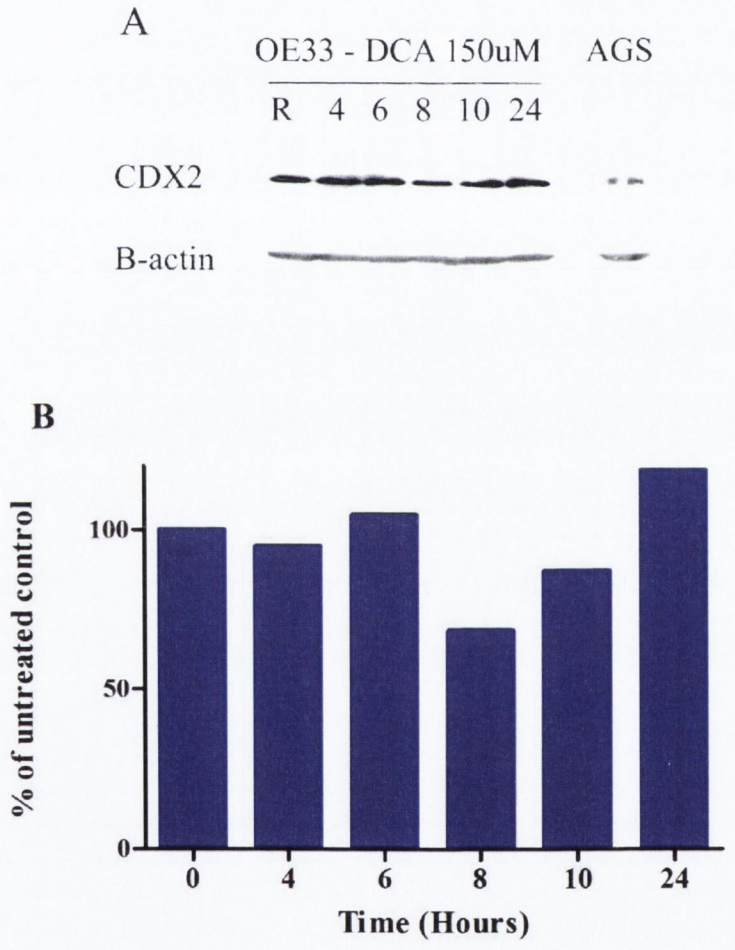


Figure 6.4: CDX2 Protein levels were decreased in response to DCA exposure [150μM] in OE33 cells. (A) Nuclear fractions from AGS cells were used as a positive control for the anti-CDX2 antibody as AGS cells express CXD2 at a high level. β-actin functioned as loading control. (B) Densitometry analysis indicated that maximum reduction of CDX2 occurred after DCA [150μM] exposure for 8 hours.

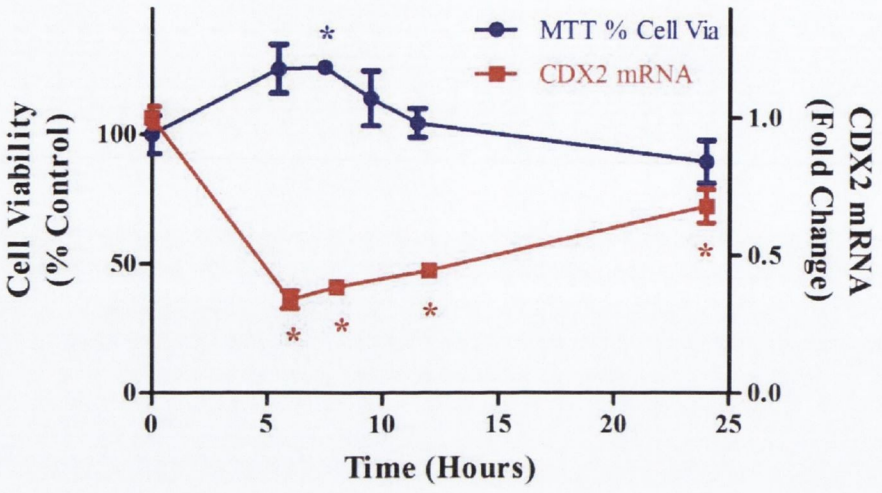


Figure 6.5: OE33 cell viability & CDX2 mRNA expression following exposure to DCA [150μM]. Cell viability of OE33 cells was not reduced following exposure to DCA [150μM] as assessed using an MTT cell proliferation assay (blue line). DCA treatment [150μM] over time reduces CDX2 mRNA expression with maximal reduction after 6 hours. Similar experimental conditions result both the lowest level of transcriptional activity of CDX2 and the highest level cell viability suggesting a role for CDX2 in reducing proliferative potential of OE33 cells in response to DCA exposure (* p-value < 0.05 compared to untreated control; Student's t-test.)

6.3.5 CDX2 & VIL1 mRNA expression in OE33 cells after exposure to a bile salt cocktail

In vivo exposure of oesophageal tissue to bile salts during an episode of reflux involves a simultaneous insult by a number of bile salts in combination with other components of refluxate. In order to recreate a system *in vitro* that more accurately represents events *in vivo,* OE33 cells were exposed to a cocktail of bile salts with similar concentrations of conjugated bile salts as observed in patients suffering with GORD (Kauer *et al.*, 1997). This cocktail was comprised of the following conjugated bile acids: glycocholic acid, taurocholic acid, glycochenodeoxycholic acid, taurochenodeoxycholic acid, glycodeoxycholic acid and taurodeoxycholic acid in a 20:3:15:3:6:1 ratio of molar concentration (Huo et al., 2010, Nehra et al., 1999, Kauer et al., 1997) OE33 cells were exposed to the bile salt cocktail in neutral medium for 6 or 8 hours and the mRNA expression of CDX2 was determined using real-time RT-PCR.

Exposure of OE33 cells to a bile acid cocktail decreased CDX2 mRNA expression to a statistically significant level (Figure 6.6). The biological significance of this reduction in CDX2 mRNA expression was confirmed by assessing the mRNA expression of a downstream target gene of CDX2, VIL1. VIL1 mRNA expression is also decreased in OE33 cells following exposure to a bile salt cocktail (Figure 6.7).

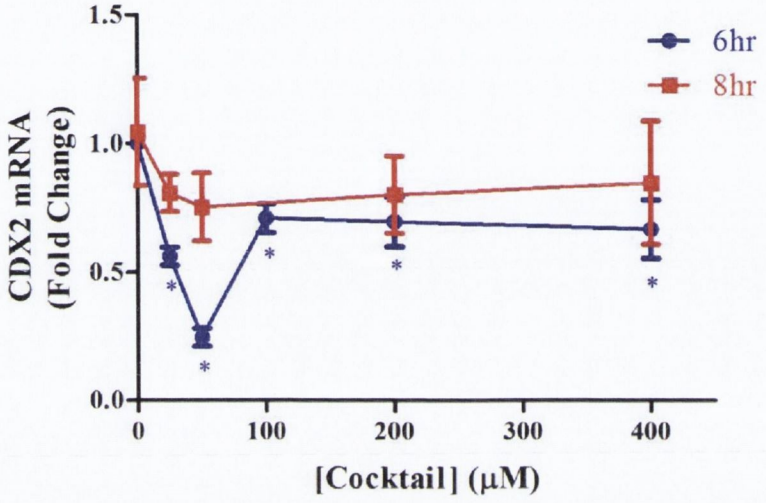


Figure 6.6: CDX2 mRNA expression is decreased in OE33 cells in response to a bile salt cocktail. CDX2 mRNA expression was reduced significantly following DCA exposure for 6 hours at all concentrations.

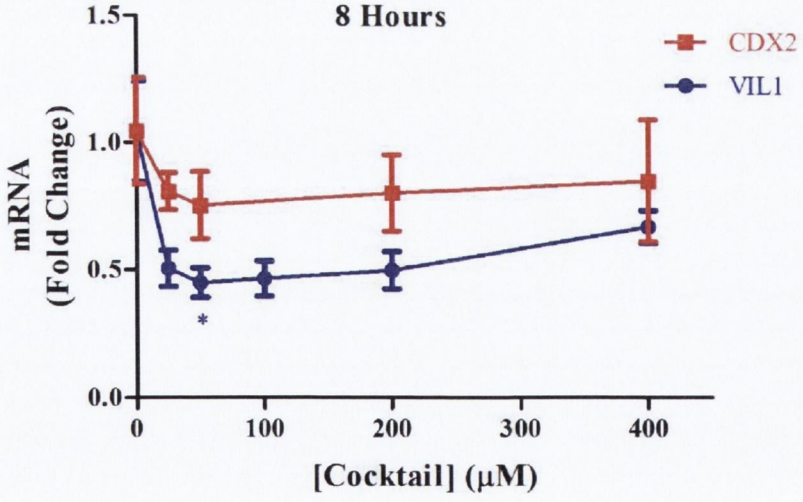


Figure 6.7: Bile salt cocktail decreased the expression of CDX2 & VIL1 in OE33 cells. CDX2 is a potent regulator of VIL1 expression. As such, the decrease in the level of VIL1 observed in OE33 cells following treatment with a bile salt cocktail for 8 hours implies that the level of functional CDX2 protein has also been decreased to a level that is biologically significant.

6.3.6 CDX2 mRNA expression following continuous and pulsatile exposure to acid and bile salts

As stated in section 6.3.5, oesophageal tissue is not exposed to single bile salts in isolation. Instead, a milieu of harmful agents are introduced during each episode of reflux which includes a cocktail of bile salts along with gastric acids which lowers the pH of the oesophagus. As such, the effect of acid, a bile salt cocktail and the combination of both on OE33 cells was determined.

Continuous exposure to a bile salt cocktail [400μM] in acidified medium (pH5.5) for 12 hours resulted in a significant decrease in CDX2 mRNA expression. A significant decrease in CDX2 mRNA expression also resulted from continuous exposure to bile salts [400μM] in neutral (pH7.4) and in acidified (pH5.5) media for 24 hours. It was also found that exposure to bile salts [400μM] in acidified (pH5.5) medium for 10 minute pulses 3 times a day for 7 days decreased CDX2 mRNA expression in OE33 cells. Expression levels of CDX2 mRNA do not deviate significantly from resting expression levels following exposure of OE33 to acidified (pH5.5) medium only (Figure 6.8).

6.3.7 Maintenance of decreased CDX2 expression in OE33 cells following exposure to pulses of a bile salt cocktail in neutral and acidified media

This study has demonstrated that exposure of the OE33 cell line to a bile salt cocktail in neutral and acidified media decreases the level of CDX2 mRNA expression. This decrease in CDX2 expression may be an important event in the development of oesophageal OAC (Vaninetti *et al.*, 2009). As such, we determined whether the decrease in CDX2 expression was maintained over time without exposure to bile and acid or if it was a transient event and expression levels were returned to basal levels if cells were allowed to rest in bile salt free complete medium at a neutral pH. Exposure to a bile salt cocktail [400μM] in neutral

(pH7.4) and acidified (pH5.5) media for 7 days with 3 pulses daily resulted in a decrease in CDX2 mRNA expression (Figure 6.8).

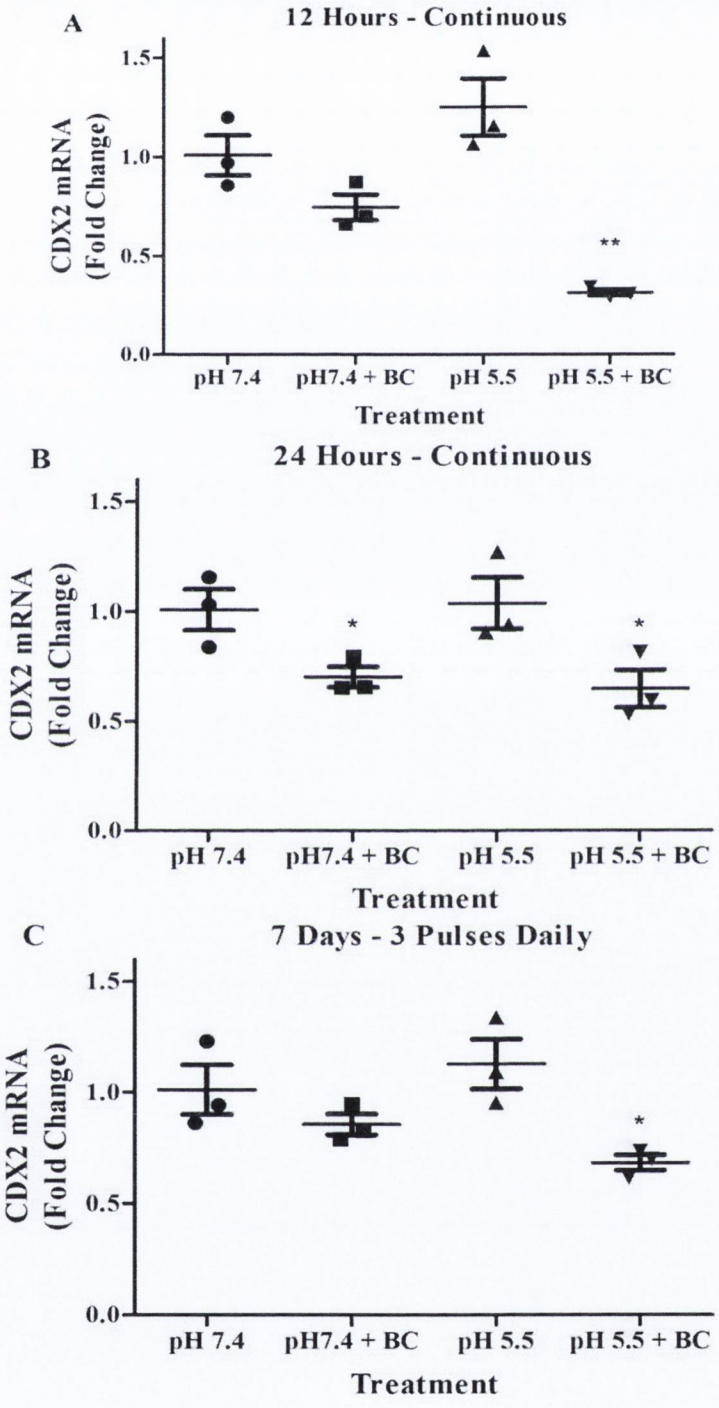


Figure 6.8: CDX2 mRNA expression is decreased following treatment with a bile salt cocktail and acidified medium in combination with a bile salt cocktail. CDX2 mRNA expression in OE33 cells is decreased following continuous treatment with a bile salt cocktail [400μM] in acidified (pH5.5) medium for 12 hours or bile salt cocktail [400μM] in neutral medium (pH7.4) and in acidified medium (pH5.5) after 24 hours. A 7 day pulse treatment with 3 ten minute pulses daily of bile salt cocktail [400μM] in acidified (pH5.5) also decreased CDX2 expression to a significant level. *p-value < 0.05; **p-value < 0.01 (Student's t-test – compared to untreated control pH7.4; BC = Bile salt cocktail).

Following the final pulsed exposure, treatment medium was replaced with bile salt free complete medium at neutral pH for 24 or 48 hours. CDX2 expression levels returned to resting levels in cells that had been treated with bile salts in neutral (pH7.4) and acidified (pH5.5) media (Figure 6.9). These data would indicate that a reduction in CDX2 expression following exposure to bile and acid is a transient event and may require repeated exposure to bile and acid to maintain decreased expression levels. This would suggest that the reduction of CDX2 expression observed during progression from BO to associated OAC requires continuous exposures to short pulses of bile and acid.

6.3.8 NF-κB inhibition ablates the transcriptional response of CDX2 in response to DCA exposure of OE33 cells

NF-κB has been shown to be an important regulator of CDX2 transcription in response to acid and bile salt exposure in other oesophageal cells lines (Huo *et al.*, 2010). As such, the contribution of NF-κB in the regulation of CDX2 expression in response to bile and acid was assessed using a chemical inhibitor of NF-κB, Bay 11-7085. Bay 11-7085 prevents NF-κB nuclear translocation through the inhibition of the TNFα-inducible phosphorylation of IκBα.

DCA decreases CDX2 mRNA expression by approximately 50% (Figure 6.10 A). Inhibition of NF-κB using the chemical inhibitor Bay 11-7085 resulted in the complete ablation of the DCA mediated decrease of CDX2 mRNA expression in OE33 cells. CDX2 expression was increased significantly following NF-κB inhibition with or without DCA. Co-treatment of OE33 cells with an NF-κB inhibitor and DCA resulted in a larger induction of CDX2 expression than treatment with the NF-κB inhibitor alone (Figure 6.10 B). This indicates that the DCA induced decrease of CDX2 mRNA expression may be mediated by NF-κB.

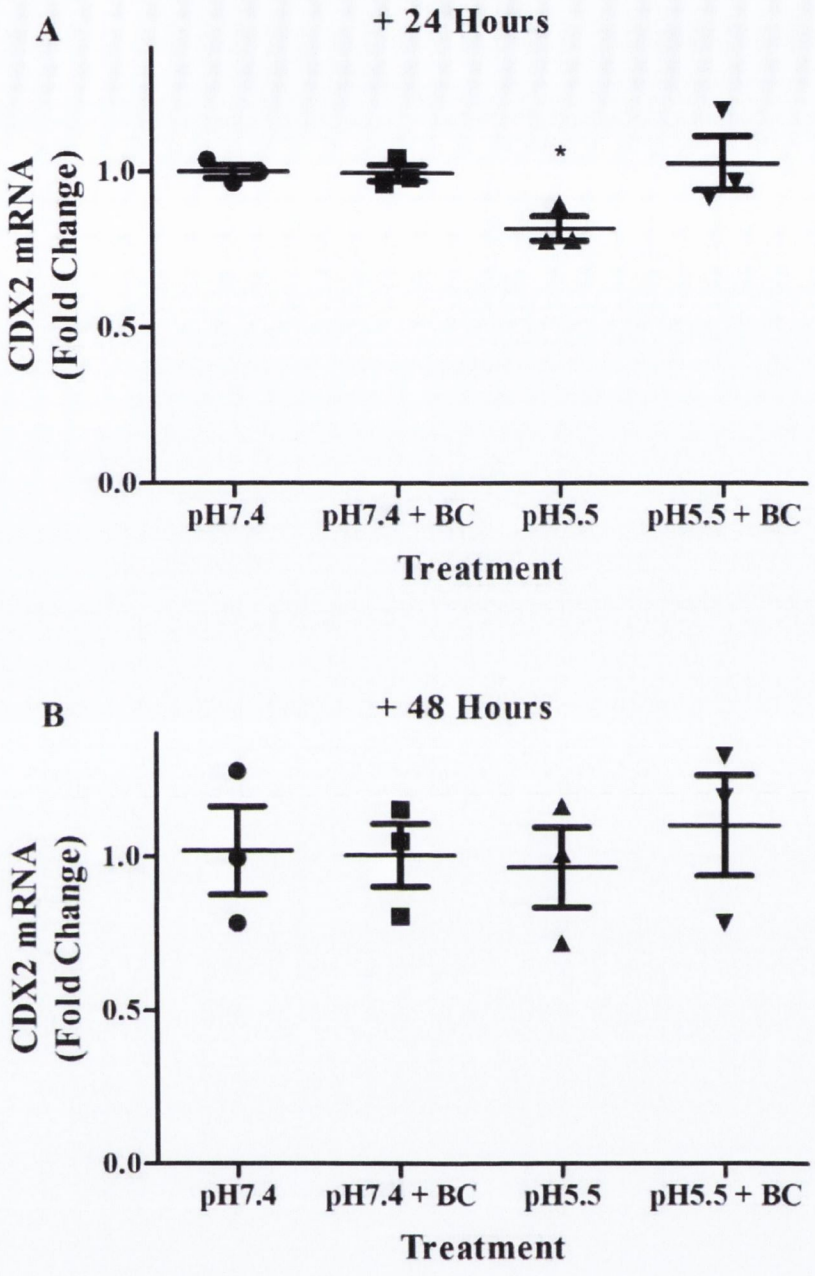


Figure 6.9: Decreased CDX2 mRNA expression in OE33 cells following 7 days pulsing with a bile salt cocktail [400μM] and/or acidified (pH5.5) medium returns to resting levels after 24 hours. Following a 7 day treatment with 3 ten minute daily pulses of bile salt cocktail [400μM], acidified medium (pH 5.5) or a combination of both, OE33 cells were allowed to rest in complete medium for 24 and 48 hours. The CDX2 levels had returned to a level similar to the untreated resting controls 24 hours post treatment and maintained these resting levels at 48 hours post treatment (* p-value < 0.05; Student's t-test – compared to untreated control pH7.4; BC = bile salt cocktail).

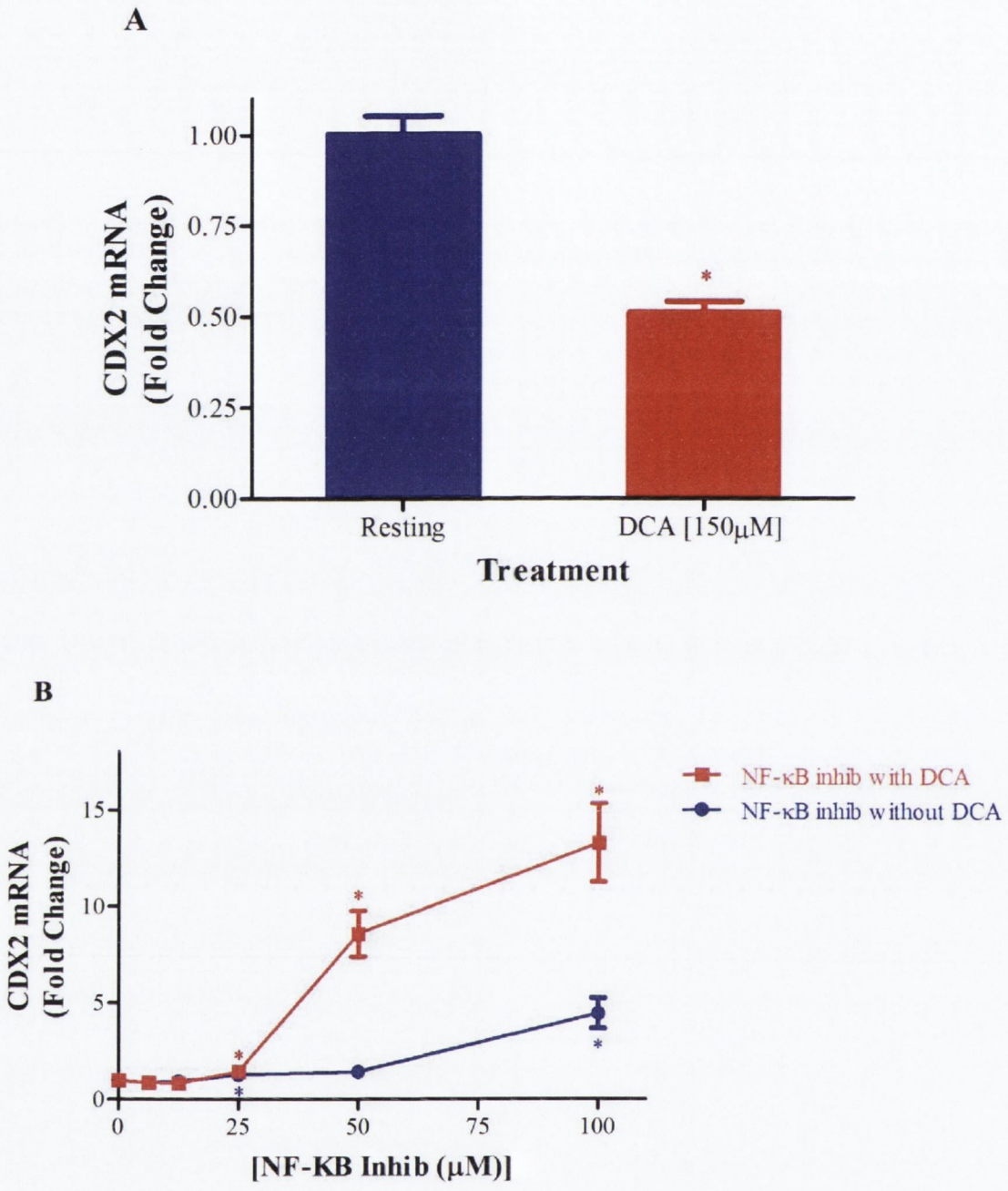


Figure 6.10: CDX2 mRNA expression decrease in OE33 in response to DCA treatment is ablated by the inhibition of NF-κB using the chemical inhibitor Bay 11-7085. (A) CDX2 mRNA expression is decreased by ~50% in OE33 cells treated with DCA ([150μM]; 8 hours). (B) This observed decrease in CDX2 mRNA expression is completely reversed in OE33 cells pre-treated with the NF-κB chemical inhibitor (Bay 11-7085, 30 minutes) followed by a co-treatment with DCA ([150μM]; 8 hours). CDX2 is increased by ~12 fold in OE33 cells as a result of NF-κB inhibition. (* = p-value < 0.05 Student's t-test.)

6.3.9 Optimisation of siRNA transfection conditions of OE33 cells

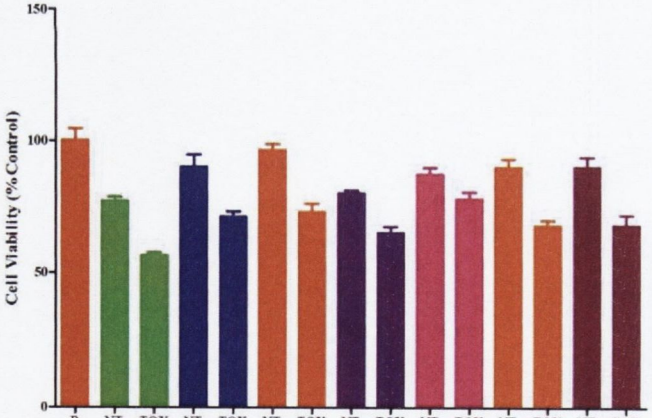
The factors that affect the efficiency of siRNA transfection include selection and quantity of transfection reagent to be used; final concentration of siRNA and quantity of transfection complex (transfection reagent and mRNA complex) in each well. These factors should be optimised for each cell line using a number of control siRNAs. Non-targeting siRNA (siNT or NT) assesses off-target effects induced by suboptimal transfection conditions. Transfection efficiency is determined using an siRNA that reduced cell viability, siTOX (TOX). Four different DharmaFECT transfection reagents are available commercially. A number of combinations of the transfection reagents described above were examined using each of the DharmaFECT reagents.

DharmaFECT 3 transfection reagent at 0.15µl per well with a final siRNA concentration of 100nM were determined to be optimal transfection conditions for OE33 cells (Figure 6.11). Further studies requiring siRNA transfections for high throughput screening involving this cell line will be carried out under these conditions.

Figure 6.11: Optimisation of siRNA transfection conditions in OE33 cells. It was determined that DharmaFECT 3 [0.15µl/well] and siRNA at a final concentration of 100nM/well were the optimum transfection conditions (highlighted by green circle). Cell viability of OE33 cells was determined using an MTT assay. Off-target effects were assessed using siNT, a non-targeting siRNA negative control that would not alter cell viability under optimal conditions. Transfection efficiency was assessed using siTOX, a positive control that will reduce cell viability under optimal transfection conditions. (R: untreated resting control; NT: siNT – non-targeting siRNA negative control; TOX: siTOX – positive siRNA control, reduced cell viability.)

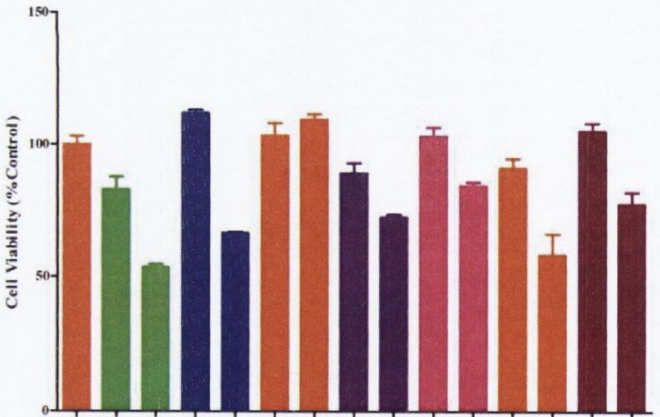
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DharmaFECT 1



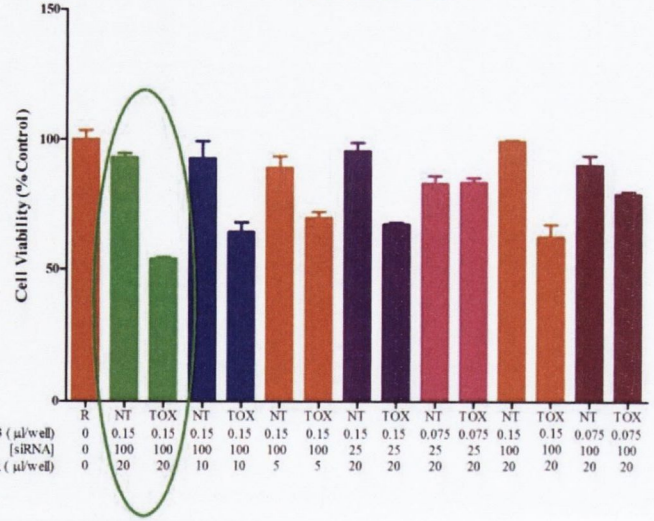
	R	NT	TOX	NT	TOX	NT	TOX	NT	TOX	NT	TOX	NT	TOX	NT	TOX
DharmaFECT 1 (µl/well)	0	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.075	0.075	0.15	0.15	0.075	0.075
[siRNA]	0	100	100	100	100	100	100	25	25	25	25	100	100	100	100
Transfection Complex (µl/well)	0	20	20	10	10	5	5	20	20	20	20	20	20	20	20

DharmaFECT 2



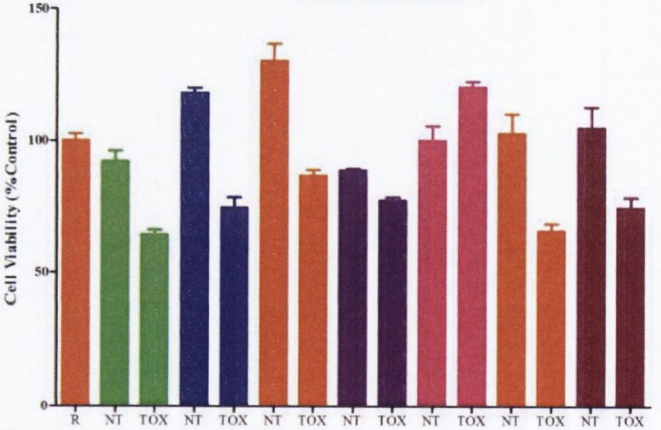
	R	NT	TOX	NT	TOX	NT	TOX	NT	TOX	NT	TOX	NT	TOX	NT	TOX
DharmaFECT 2 (µl/well)	0	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.075	0.075	0.15	0.15	0.075	0.075
[siRNA]	0	100	100	100	100	100	100	25	25	25	25	100	100	100	100
Transfection Complex (µl/well)	0	20	20	10	10	5	5	20	20	20	20	20	20	20	20

DharmaFECT 3



	R	NT	TOX	NT	TOX	NT	TOX	NT	TOX	NT	TOX	NT	TOX	NT	TOX
DharmaFECT 3 (µl/well)	0	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.075	0.075	0.15	0.15	0.075	0.075
[siRNA]	0	100	100	100	100	100	100	25	25	25	25	100	100	100	100
Transfection Complex (µl/well)	0	20	20	10	10	5	5	20	20	20	20	20	20	20	20

DharmaFECT 4



	R	NT	TOX	NT	TOX	NT	TOX	NT	TOX	NT	TOX	NT	TOX	NT	TOX
DharmaFECT 4 (µl/well)	0	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.075	0.075	0.15	0.15	0.075	0.075
[siRNA]	0	100	100	100	100	100	100	25	25	25	25	100	100	100	100
Transfection Complex (µl/well)	0	20	20	10	10	5	5	20	20	20	20	20	20	20	20

6.4 Discussion

Bile salts have been implicated in several studies as modulators of CDX2 expression. CDX2 is important in oesophageal carcinogenesis as induction of this transcription factor has been identified as an early event in normal squamous transformation to intestinal metaplasia (Hu *et al.*, 2007). Target genes of CDX2 are involved in cell processes that can be associated with carcinogenesis such as proliferation, differentiation and apoptosis (Groisman *et al.*, 2004). This body of work aimed to develop an assay that would be applicable in an siRNA library screen to identify modulators of alterations in CDX2 expression as a result of exposure to bile salts.

It was observed that the basal levels of CDX2 expression differs greatly between cell lines with origins in different tissues and different disease stages (Figure 6.1). The OE33 cell line, derived from a pathological stage IIA oesophageal tumour, expressed a high level of CDX2 mRNA when compare to normal oesophageal squamous cells (~400 times greater). An increase in CDX2 expression has been demonstrated during the development of BO (Phillips *et al.*, 2003). The ability of bile salts to induce CDX2 mRNA expression in Het-1A normal oesophageal cells was assessed. OE33 cells were the only oesophageal cell line examined that display a basal level of CDX2 mRNA expression (Figure 6.1). Further work was carried out on OE33 cells to assess their response to bile salt exposure. As OE33 exhibited a basal level of CDX2 expression, an induction or a suppression of CDX2 activity could be determined.

CDX2 expression is known to increase as normal squamous cells become metaplastic (Phillips *et al.*, 2003) (Figure 6.12). The response of CDX2 mRNA expression in Het-1A cells to bile salt and acid exposure was examined (Figure 6.2). CDX2 mRNA expression was induced following exposure to a bile salt cocktail [400μM] in neutral (pH7.4) and

acidified (pH5.5) media in initial experiments to varying degrees. The induction of CDX2 cells, however, was inconsistent. This may be a result of a low copy number of the target gene in the initial sample which results in Ct values that are close to the maximum number of cycles that the PCR reaction undergoes during this protocol. This suggests that CDX2 detection is a very late event and that CDX2 mRNA expression levels are extremely low in these samples. A pre-amplification step may have facilitated the detection of alterations in CDX2 expression in the Het-1A cells in response to DCA exposure. This technique was not utilised in this study as the alterations in CDX2 expression in OE33 cells as a result of stimulation with DCA were further investigated.

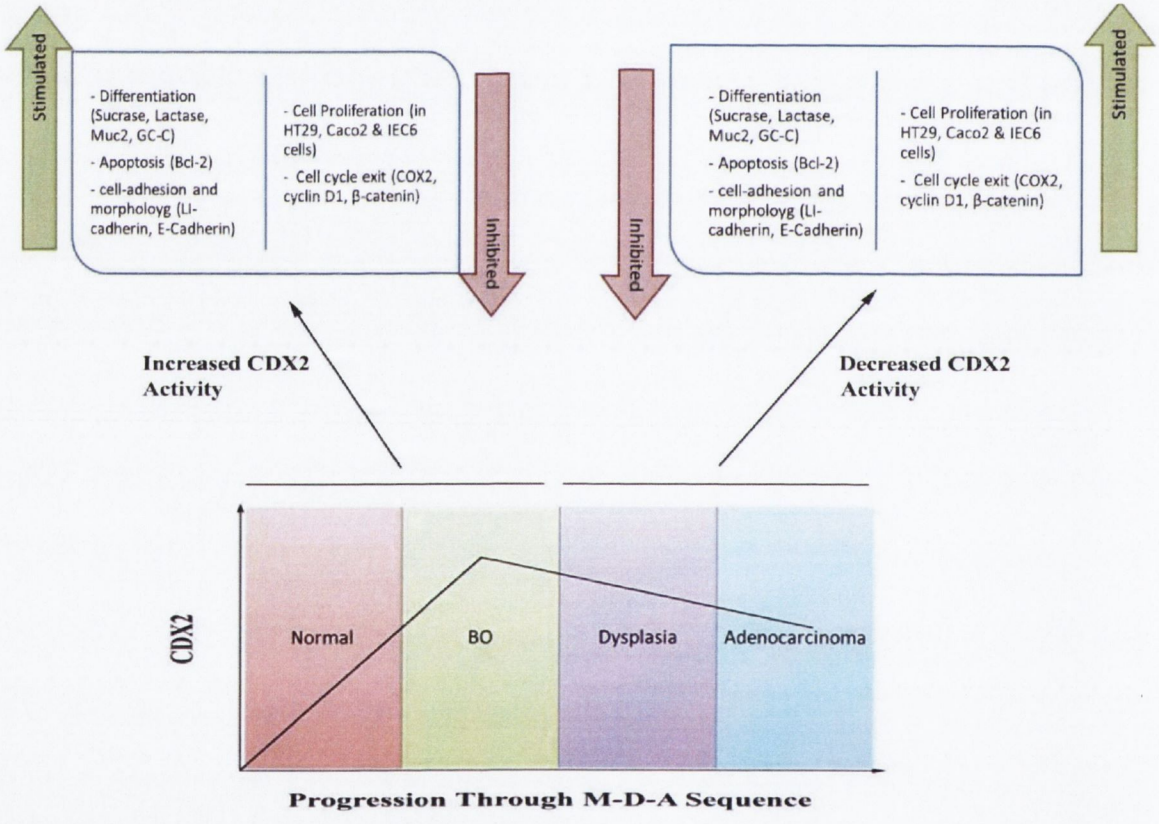


Figure 6.12: CDX2 expression is lost in progression from BO to OAC and may act as a tumour suppressor gene. CDX2 expression is upregulated in the development of intestinal type tissue in the oesophagus but expression is lost as BO develops into OAC. CDX2 has been suggested to act as a tumour suppressor in colon cancer due to the fact that CDX2 expression is markedly reduced in a subset of colon cancers. CDX2 expression is also associated with a decrease in proliferative potential and an increase in cell differentiation in colon cancer cell lines. CDX2 expressing cells are also more susceptible to apoptosis. COX2, which is known to have elevated expression in colon cancers, is inhibited by CDX2 (Kim et al., 2004, Suh and Traber, 1996, Phillips et al., 2003, Vaninetti et al., 2009).

The response of Het-1A cells following exposure to bile salts and acid resulted in an increase of CDX2 expression. CDX2 copy numbers in both resting and stimulated cells were quite low. An increase in the number of days of exposure of the cells may result in a greater level of induction of CDX2 expression. The process of pulsing cells three times daily is quite abrasive as there are six media changes per day. In the case of Het-1A cells, attempts to lengthen the treatment time resulted in the washing off of the cells from the treatment wells. Optimisation of the experimental process involving a less abrasive protocol would facilitate the expansion of this experiment. A reduction in the force used to aspirate and dispense the media would be key to preventing the loss of cells as a result of experimental processes rather than from the experimental conditions that are under scrutiny. Other methods that may be employed to assess levels of CDX2 induction following bile acid exposure include the introduction of a luciferase linked CDX2 promoter or the introduction of a GFP linked CDX2 promoter to the Het-1A cells.

CDX2 expression levels have been found to decrease in progression from Barrett's metaplasia through the various grades of dysplasia to OAC (Phillips *et al.*, 2003) (Figure 6.12). A potential mechanism of this process is the negative regulation of CDX2 by HIF-1α (Zheng et al., 2010). HIF-1α expression is negatively correlated with the expression of CDX2 throughout the oesophageal carcinogenic sequence. As CDX2 expression increases in the development of BO, HIF-1α expression decreases. During progression to OAC, HIF-1α increases as CDX2 increases (Griffiths et al., 2007). Further experimental evidence is required to establish if these differences in expression levels are significant in CDX2 regulation and oesophageal carcinogenesis. It has been demonstrated in this study that exposure of OE33 cells to unconjugated deoxycholic acid or a bile salt cocktail reduces CDX2 expression (Figure 6.3, Figure 6.6, Figure 6.8). These results indicate that exposure of OE33 cells to bile acids during an episode of reflux may result in a decrease in

CDX2 expression. In colon carcinogenesis, CDX2 expression is greatly reduced in the later stages of carcinoma development, indicating a potential tumour suppressor role for CDX2 (Ee *et al.*, 1995, Bonhomme *et al.*, 2003). Further evidence of a role for CDX2 as a tumour suppressor gene in colon cancer arises from two mouse studies. Bonhomme *et al* (2003) demonstrated that mice heterozygous for a functional CDX2 allele ($CDX2^{+/-}$), treated with a carcinogen, develop tumours in the distal colon 12 weeks after treatment with the carcinogen. Wild-type litter-mates who had undergone similar treatment did not develop any tumours suggesting that two functional CDX2 alleles are sufficient to prevent tumour development (Bonhomme *et al.*, 2003). A second mouse study demonstrated that double heterozygous animals for the CDX2 and the APC alleles develop approximately six times more colonic polyps than animals with either a CDX2 or APC mutant allele only (Aoki *et al.*, 2003). If CDX2 does function as a tumour suppressor gene in oesophageal OAC development from BO, the decrease observed in this study of CDX2 expression in response to bile acid exposure would have important consequences (Figure 6.12). Identifying the signalling pathways involved in this process has the potential to identify novel therapeutic strategies targeting prevention of progression from BO to OAC. The implementation of an siRNA library screen which targets well known signalling pathways may reveal modulators of CDX2 suppression in response to bile acid exposure.

The mRNA expression of a downstream target gene of CDX2, VIL1 was also decreased significantly. Interestingly, the data indicates that the decrease in VIL1 expression occurs shortly after the introduction of DCA to the cells before a significant decrease in the level of CDX2 mRNA is detected (Figure 6.3). The immediate reduction in the expression level of the CDX2 target gene Villin suggests that exposure to bile acids reduced the functionality of CDX2 as well as reducing CDX2 expression. The observed reduction in the levels of Villin expression occurs prior to a significant decrease in CDX2 expression

suggesting that the CDX2 available within the cell is rendered inactive before CDX2 expression is decreased. CDX2 functions as an auto-regulator through an AT rich site designated the downstream binding site (Xu *et al.*, 1999). A reduction in functional CDX2 would contribute to a decrease in the mRNA expression of target genes which include CDX2 itself, leading to a reduction in CDX2 mRNA expression as was observed in this study (Figure 6.3). The activation domain of CDX2 has been shown to be phosphorylated resulting in a decrease in CDX2 activity (Rings *et al.*, 2001). Inactivation of CDX2 as a consequence of phosphorylation would result in reduction in activity and a consequent decrease in expression of target genes of CDX2.

As the response of OE33 cells to DCA was the most consistent and significant effect observed, further work to develop a semi-automated siRNA screen examining DCA signalling in oesophageal cells will be carried out using the OE33 cell line. An important element of any high throughput/high content screen is the development of relevant positive controls to assess the efficiency of siRNA transfection on a larger scale. NF-κB has previously been identified as a regulator of CDX2 expression in response to bile acid exposure and as such is an ideal candidate to develop as a positive control in an siRNA library screen (Huo *et al.*, 2010, Kazumori *et al.*, 2006). A chemical inhibitor of NF-κB, Bay 11-7085, was used to assess NF-κB functionality in this cell system. The mRNA levels of CDX2 in OE33 cells were determined after a pre-treatment with the chemical inhibitor followed by a cotreatment with DCA and a chemical inhibitor (Figure 6.10). NF-κB inhibition, in conjunction with DCA exposure resulted in an induction of CDX2 mRNA expression. In contrast, DCA exposure in the absence of the NF-κB inhibitor resulted in the suppression of CDX2. This would indicate that NF-κB is involved in the suppression of CDX2 expression by DCA exposure and as such is a potential positive control for an siRNA library screen.

Preliminary experiments involving the response of OE33 cells to bile acid exposure indicate that this *in vitro* system represents a powerful tool to elucidate the mechanisms by which bile salts affect gene expression. The siRNA transfection conditions have been optimised; however, the transfection process remains to be completely upscaled to the level required for large scale high throughput screening processes using liquid handling robots. An interesting siRNA screen to execute would be an investigation into the upstream regulators of CDX2. It has been postulated, based on data obtained from a study involving somatic cell hybrid experiments, that suppression of CDX2 transcription is subject to a trans-acting regulatory mechanism but the identity of the regulator remains unknown. This study was carried out in colon cancer cell lines with the aim to gain further insights into the role of CDX2 in colorectal cancer as previous studies have demonstrated that CDX2 in downregulated in some colon cancers (Hinoi *et al.*, 2003). Preliminary experiments in this study have demonstrated that both an increase in OE33 cell viability and a decrease in CDX2 expression occur under similar experimental conditions (Figure 6.5). These data suggest that in the OE33 cell line, CDX2 may suppress cell proliferation. An siRNA screen to identify upstream regulators of CDX2 expression could be based on the proliferative potential of OE33 cells following specific inhibition of a library of siRNAs. Initial experiments are required to confirm that inhibition of CDX2 would result in an increase in proliferative potential of the cell line.

NF-κB has previously been identified as a potential suppressor of CDX2 activity using a chemical inhibitor Bay 11-7085 (Figure 6.10). Two NF-κB binding sites which facilitate regulation of CDX2 transcription by NF-κB have been identified in the CDX2 promoter (Kazumori *et al.*, 2006). Confirmation of NF-κB functionality as a suppressor of CDX2 transcription using an siRNA identifies a potential regulatory mechanism for CDX2 transcription and would also demonstrate a potential function for NF-κB as a positive

control in a high-throughput siRNA library screen. The screen could utilise the previously optimised semi-automated reverse transfection protocol in conjunction with high-throughput MTT assay to identify regulators of CDX2 expression. Identification of these regulators would be important as there is evidence to suggest that CDX2 has a tumour suppresser function in the colon and possibly also in the oesophagus. Interfering with the process by which CDX2 suppression during development of OAC from BO may be a potential therapeutic strategy for cancer progression prevention. Signalling pathways identified as being involved in CDX2 regulation may also be involved in the hypothesised switch by NF-κB from promoting CDX2 in the development of BO to suppression of CDX2 in development of OAC from BO.

NF-κB has previously been demonstrated to control induction of CDX2 in HT-29 cells in response to dehydrocholic acid (DHCA) and cholic acid (CA) exposure (Huo *et al.*, 2010, Kazumori *et al.*, 2006). NF- κB mediated induction of CDX2 was also demonstrated in normal oesopahgeal cells derived from GORD patients with associated BO (Huo *et al.*, 2010). In this study, NF-κB has been described as a potential suppressor of bile acid-induced CDX2 in a cell line representing the later stages of the M-D-A sequence i.e. during progression from BO to OAC. It is known that CDX2 expression levels decrease in the later stages of oesophageal carcinogenesis (Phillips *et al.*, 2003) (Figure 6.12). Data described in this chapter demonstrated that NF-κB mediated inhibition of CDX2 may represent an altered NF-κB regulatory mechanism. While NF-κB is observed to display increased DNA binding throughout the oesophageal carcinogenic sequence (Abdel-Latif *et al.*, 2009), these experiments may indicate that its target genes and functionality may differ at different stages of the oesophageal carcinogenic sequence. Kim *et al* (2002) have demonstrated that the p50/p50 homodimer and the p65/p50 heterodimer exert opposing effects on CDX2 expression: binding of the p65/p50 heterodimer to the CDX2 promoter

supresses CDX2 expression whereas the p50/p50 homodimer induces CDX2 expression (Kim *et al.*, 2002). It is proposed that a switch occurs in the underlying regulatory mechanism of NF-κB at the point where BO mucosa becomes malignant and transforms into dysplastic and subsequently OAC tissue. NF-κB is expressed throughout the M-D-A sequence whereas CDX2 expression increases during normal to BO progression and decreases during development of OAC from BO. Regulation of CDX2 by NF-κB is dependent on the preferential binding of NF-κB subunits to the CDX2 promoter. It is hypothesised that a regulatory switch occurs upstream of NF-κB which causes the p50/p50 homodimer to be replaced on the CDX2 promoter by the p65/p50 heterodimer (Figure 6.13). This results in the suppression of CDX2 transcription which may contribute to the progression of carcinogenesis as CDX2 is suspected to function as tumour suppressor gene as described previously based on studies in colon cancer.

Development of this study will involve an investigation into the effect of bile salt exposure of OE33 on the nuclear translocation of both NF-κB subunits and also to identify which subunit preferentially binds the CDX2 promoter more and therefore dictates whether CDX2 expression is induced or suppressed. Identifying the mechanisms involved in the determination of the NF-κB subunit with preferential DNA binding at various stages of the carcinogenic sequence would be extremely important. Novel therapeutic targets could be identified which could be developed to treat the progression to OAC in Barrett's oesophagus patients by targeting NF-κB mediated control of CDX2 expression. Specifically identifying and targeting the possible switch in NF-κB regulation from induction of CDX2 to suppression of CDX2 may allow prevention of OAC development. An siRNA screen to assess the regulatory mechanism involved in selecting which subunit is more dominant could be developed. Targeted inhibition by siRNA of a library of genes could pinpoint signalling pathways involved in NF-κB regulation. This screen would be

more complex than previously designed screens using a single cell proliferation assay e.g. MTT assay.

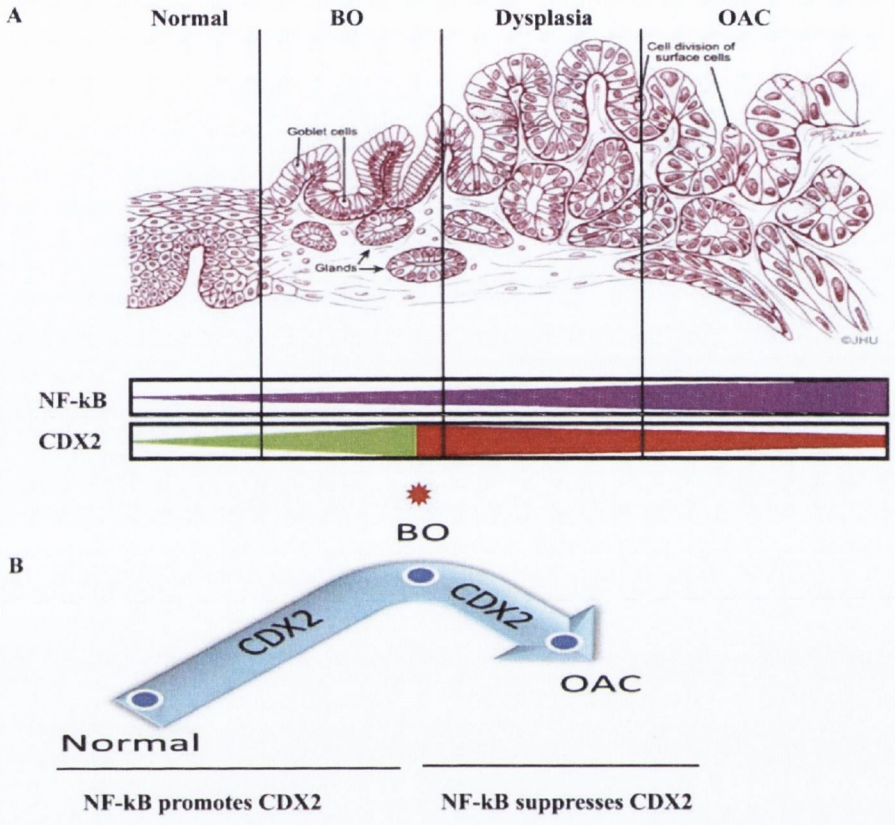


Figure 6.13: NF-κB expression increases throughout the entire M-D-A sequence whereas CDX2 expression rises from normal to BO but decreases from BO to OAC. (A) NF-κB expression increases through the M-D-A sequence from normal to OAC tissue whereas CDX2 expression increases from normal to BO mucosa but decrease from BO to OAC. (B) NF-κB is capable of involvement in the transcriptional regulation of CDX2. As BO mucosa develops, NF-κB promotes the expression of CDX2 through binding of the p50/p50 homodimer to the CDX2 promoter at one of two NF-κB specific binding sites. It is hypothesised that at approximately the point where the premalignant BO mucosa progresses into dysplastic and OAC tissue (represented by the red star), the transcriptional regulation of NF-κB causes a switch in the dominant DNA binding units of NF-κB. The p65/p50 heterodimer replaces the p50/p50 homodimer binding to the CDX2 promoter, causing a suppression of CDX2 expression. Identification of the underlying mechanism which facilitates NF-κB to switch from being a promoter to a suppressor of transcription CDX2 could present novel therapeutic strategies for the prevention of development of OAC from BO mucosa (*Modified from (Koppert et al., 2005)).*

A high content approach implementing a nuclear translocation assay may be sufficient to determine if either the p50 or p65 subunits are preferentially translocated. In the event that

both subunits translocate to the nucleus but the dominant DNA binding subunit switches, a more complex assay will need to be designed which may be too complex for high content screening. An electrophoretic mobility shift assay (EMSA) would be a method of determining DNA binding of both subunits but would not be suitable for a high throughput/high content study. However, other high content screens investigating CDX2 regulatory mechanisms may indicate a smaller panel of potential signalling pathways which could be incorporated into an EMSA study to determine of a switch in preferential binding of different NF-κB subunits is possible by altering signalling pathways.

This study for the first time demonstrates that the reduction in CDX2 expression as BO progresses to oesophageal carcinogenesis may be induced by DCA and regulated by a NF-κB. NF-κB is also a candidate to act as a positive control in an siRNA library screen to identifying modulators of CDX2 suppression in OE33 cells in response to DCA.

Chapter 7

General Discussion

7 General Discussion

Barrett's oesophagus (BO) is a pre-malignant condition associated with gastro-oesophageal reflux disease (GORD) and is a recognised precursor to oesophageal adenocarcinoma (OAC) (Jankowski *et al.*, 1999). The involvement of bile acids in BO and OAC development is well established, however, the pathogenesis of BO is still somewhat unclear, as is the exact mechanisms by which bile acids affect normal cellular mechanics. Oesophageal adenocarcinoma is an extremely aggressive disease with a poor prognosis (Sundelof *et al.*, 2002). Surgical resection for early-stage disease is a curative treatment option, however for patients that do not have this option; further treatment possibilities are somewhat limited. The development of new therapeutic approaches has the potential to offer a much more effective treatment to a wider cohort of patients (Arrington *et al.*, 2009). The elucidation of mechanisms involved in bile acid signalling during the development of OAC will lead to the development of novel therapeutic strategies as new therapeutic targets are identified. The main aim of this study was to identify novel therapeutic strategies in the treatment of oesophageal adenocarcinogenesis through the development of high throughput siRNA screening approaches.

Gene expression microarray technology is a powerful method to reveal the underlying genetic changes involved in disease progression. Cellular responses to exogenous stimuli can also be detected using this technology. This thesis has developed and implemented a comparative analysis strategy for use in microarray studies. This strategy involved the integration of previously published patient derived data charting progression through the M-D-A sequence with data generated in an original *in vitro* study assessing gene expression alterations in cell lines following exposure to DCA. A cohort of DCA responsive genes was identified that were also involved in progression through the carcinogenic sequence. Approximately one third of the genes involved in oesophageal

cancer development were identified as DCA responsive, supporting the role of bile acids in oesophageal carcinogenesis. Systems biology analysis identified cell death and cell proliferation as important processes that were associated with this cohort of genes.

In order to expand on these findings, a functional siRNA high throughput screening protocol was developed to further examine the cellular processes involved in oesophageal disease progression. A semi-automated siRNA transfection protocol was established using an MTT cell proliferation assay as a robust output suitable for high throughput screening. At this point, two potential siRNA library screening strategies were under development. The first screen aimed to investigate the responses of oesophageal cell lines to DCA exposure. An siRNA screen was developed to identify modulators of resistance to DCA induced cell death that was observed in dysplastic oesophageal cells. BCL-XL was developed as a positive control for the screen following a mini screen targeting a panel of chemical inhibitors. The second screen that was developed investigated the proliferation of dysplastic oesophageal cells in the absence of external stimuli. A panel of siRNA inhibitors was established targeting oesophageal malignancy, proliferation and apoptosis related genes to identify a potential positive control for use in this screen. A combination of siRNA pools targeting BCL-XL, GATA6 and MCL1 was selected for further study as the coordinated inhibition of these three molecules was demonstrated to reduce the cell viability of GOhTRT cells by ~55%.

The siRNA screen investigating the resistance to cell death of GOhTRT cells was completed using a library of siRNA pools target G-protein coupled receptors (GPCRs). The GPCR library was chosen as these molecules represent 15% of the druggable genome and 25% of current viable experimental and marketed drugs (Hopkins and Groom, 2002). The positive targets of the screen were ranked based on cell viability and z-score. The top

targets to be identified include interleukin 8 receptor alpha (IL8Rα), interleukin 8 (IL8), glucagon-like peptide 1 receptor (GLP1R) and frizzled homolog 1, 3 & 10 (FZD 1, FZD3, and FZD10). Systems biology analysis revealed processes and pathways that were linked to the top positive targets. These processes include p53 signalling, the inflammatory response and cell death. This body of work has contributed greater insight into the survival mechanisms employed by malignant oesophageal cells. Further validation may identify novel therapeutic targets for the treatment of oesophageal disease.

A third siRNA screen was developed to investigate CDX2 homeostasis and inducibility in oesophageal cells. The expression of CDX2 during the development of BO is well established (Groisman *et al.*, 2004) however recent evidence suggests that CDX2 expression decreases as BO progresses to OAC (Vaninetti *et al.*, 2009). This study has demonstrated that exposure of the oesophageal cell line OE33 to bile acid results in a significant decrease in CDX2 expression. This decrease in CDX2 expression appears to be mediated by NF-κB, as demonstrated by inhibition of NF-κB using the chemical inhibitor Bay 11-7085. This may be important as CDX2 has been suggested to function as a tumour suppressor in colon cancer studies (Bonhomme *et al.*, 2003, Aoki *et al.*, 2003). If a similar role for CDX2 was identified in oesophageal carcinogenesis, this may present a novel therapeutic strategy to target the progression from BO to OAC. Further validation of this process, coupled with an siRNA library screen investigating modulators of this suppression of CDX2 in OE33 cells in response to bile acid exposure could identify potential novel therapeutic targets.

The silencing of target gene expression has been demonstrated in this study as a powerful *in vitro* research technique for characterising the contribution of a single gene to a phenotype through specific inhibition of that gene using siRNA technology. Another

extremely important and powerful application of RNAi technology is the silencing of disease causing genes as a therapeutic strategy. While the ability of RNAi to effectively silence target genes is not in question, there are a number of other hurdles to be overcome before a safe and effective treatment option involving RNAi is available. The main issues concerning the implementation of RNAi as a therapeutic strategy are associated with inefficiencies in the delivery systems. Naked siRNA is quite large and is also negatively charged which prevents successful entry to the cell through the cell membrane. Naked siRNA is also targeted for enzymatic degradation and/or is quickly subjected to renal clearance (Whitehead *et al.*, 2009). In order to overcome this issue, attempts have been made to chemically modify the siRNA or encase the siRNA in viral or non-viral carriers (Shim and Kwon, 2010).

An interesting attempt to overcome the problem of siRNA delivery involves the use of nanocarriers as reviewed by Weinstein *et al* (2010). There are a number of factors that need to be considered in the development of nanocarriers as a delivery method for RNAi. Nanocarriers should be biodegradable to avoid accumulation of toxic nanoparticles and they should also act specifically on the targeted disease tissue. Interestingly, nanoparticles tend to accumulate in cancerous or highly inflamed tissue which could be quite advantageous if manipulated effectively to transport RNAi molecules directly to these sites. It is also proposed that nanocarriers are equipped with antibodies or ligands that are specific to disease tissue to avoid off-target effects through binding with normal, healthy tissue (Weinstein and Peer, 2010). The design of chemically synthesised siRNA molecules for use as therapeutics is an extremely complex process. Off-target silencing or toxicity as a result of badly designed siRNA molecules must be avoided. Chemically synthesised siRNA molecules generally contain a number of modifications to ensure that the correct strand of the double stranded RNA is loaded into the RISC complex and used as a template for the targeted degradation of the appropriate gene of interest. The incorporation of 2'-O-

methyl groups into the guide and passenger strand ensure that the correct strand is loaded into the RISC complex by manipulating the thermodynamics of each strand. These modifications ensure a bias towards the incorporation of the guide strand into the RISC complex (Davidson and McCray, 2011).

There have been a number of successful studies *in vivo* where siRNA molecules have been successfully delivered and the target gene effectively inhibited in a mouse system. Shen *et al* (2006) successfully targeted *vegfr1* mRNA in the eye of the mice using siRNA with stabilisation modifications. Intravitreous and periocular injections were used to deliver the siRNA directly to the area of interest which resulted in a statistically significant decrease in the levels of mRNA expression of the target gene (Shen *et al.*, 2006). Systemic delivery using cholesterol modified cell-penetrating peptides has been successfully achieved by Crombez *et al* (2009). The bioavailability and stabilisation of the peptide/siRNA nanoparticles was greatly improved using a cholesterol coating. Alexa^{700} labelled siRNA was used to demonstrate an accumulation of siRNA mainly in the lung, liver, plasma, skin, kidney, adrenal gland and spleen. Intravenous injection of siRNA targeting cyclin B1 effectively reduced the size of xenografted tumours in mice. This reduction in tumour size directly correlated to a reduction of cyclin B1 protein level (Crombez *et al.*, 2009). These advances are extremely promising in the quest to establish RNAi as a viable therapeutic strategy.

There are still considerable obstacles to be overcome in the development of robust and effective RNAi delivery techniques; however, considerable progress has been made in the last decade in this area. Meanwhile, preliminary studies investigating the ability of RNAi to inhibit tumour growth *in vivo* have been undertaken. This strategy involves the comparison of the growth of xenografted tumours with and without pre-transfection with RNAi molecules targeting genes of interest. Arrington *et al* (2009), demonstrated that

specific inhibition of ERBB2 following siRNA targeted inhibition resulted in a decrease in the cell viability of an oesophageal adenocarcinoma cell line, OE19 *in vitro*. Expanding on these data, OE19 cells were pretransduced with lentiviral short hairpin (shRNA) targeting ERBB2. Oesophageal adenocarcinoma xenografts were established in female *nu/nu* nude mice. Mice injected with OE19 cells pre-transfected with siRNA targeting ERBB2 demonstrated a significantly slower rate of tumour growth which corresponded to a significant suppression in the protein levels of ERBB2. By using this technique, *in vivo* data has been demonstrated that ERBB2 suppression can inhibit tumour growth in upper GI adenocarcinomas (Arrington *et al.*, 2009). Wang *et al* (2009) implemented a similar approach to demonstrate that inhibition of β-catenin in an oesophageal carcinoma suppresses tumour growth (Wang *et al.*, 2009). Implementation of this type of system could provide valuable preliminary *in vivo* information regarding novel therapeutic targets and their ability to suppress actual tumour growth.

The development of a robust RNAi delivery system is needed to progress these animal studies to a level that is closer to a transition from animal models to clinical trials in patients. There are currently a number of techniques that successfully induce BO like lesions in animal models. This process involves the anastomosis of the duodenum directly to the oesophagus with or without gastric preservation depending on the requirement of gastric components for the study design. This technique to induce reflux has been shown to successfully induce metaplastic tissue in the oesophagus (Pera *et al.*, 2000) and *de novo* expression of Cdx2 in the oesophagus (Ingravallo *et al.*, 2009). Of relevance to this study, Naito *et al* (2006) identified an important role for trypsin in the induction of oesophageal mucosa damage by using rats that had undergone oesophagogastroduodenal anastomosis in conjunction with treatment with a variety of inhibitors (Naito *et al.*, 2006). Following the development of functional siRNA delivery systems, the methods employed in these publications could be incorporated into a study to determine the feasibility of RNAi as a

novel therapeutic strategy in the treatment of oesophageal malignancies. In the interim, as RNAi delivery strategies are optimised, these animal models could be utilised to assess currently available small molecule inhibitors and other chemical inhibitors as potential therapeutic strategies.

This study has established a number of avenues for future investigations in the development of a novel therapeutics for the treatment of oesophageal adenocarcinoma and associated premalignant conditions. The combined targeted inhibition of BCL-XL, GATA6 and MCL1 results in the substantial decrease in the cell viability of a dysplastic oesophageal cell line. This inhibition is an ideal candidate as a positive control in an siRNA screen. Also, these data alone compels further investigation as there may be a novel application in the treatment of oesophageal disease for previously developed small molecule inhibitors which are currently in clinical trials for other malignancies including small-cell lung cancer and chronic lymphocytic leukaemia (Vogler *et al.*, 2009). Further preliminary work is required to validate that the decrease in cell viability is a result of specific inhibition of BCL-XL and MCL1 and not a result of off-target effects. A simple assessment of the mRNA and protein expression levels of both molecules following siRNA treatment would confirm that the reduction in viability is a result of the specific inhibition of the intended molecules. An apoptosis assay examining mitochondrial membrane potential, pro-apoptotic caspase levels and/or nuclear condensation would establish whether the reduction in cell viability is a result of induction of apoptosis. Alternatively, the reduction in viability may be a result of cell cycle arrest and should also be examined. It would also be important to investigate if this mechanism is specific to dysplastic cells. *In vitro,* specific inhibition of these molecules using combined siRNA pools in a number of cell lines charting the oesophageal carcinogenic sequence would give a greater insight into the specificity of this novel therapeutic strategy. Validation experiments as described for

the dysplastic line would be important for all cell lines to confirm that the phenotype being characterised is a result of specific inhibition. The progression to an *in vivo* study could utilise both the xenograft and the anastomosis animal models described previously to assess the potential of small molecule inhibitors targeting these anti-apoptotic molecules (Vogler *et al.*, 2009).

Protein expression levels are also subject to influence and control from non-coding RNA species. A small number of studies have been recently published which investigate the role of microRNAs (miRNAs) in the development of Barrett's oesophagus and oesophageal adenocarcinoma. These studies have made significant progress towards the discovery of an miRNA gene expression profile with the potential to act as a predictor of disease progression (Feber et al., 2008, Yang et al., 2009). More specifically, individual miRNAs have also been identified with altered expression profiles during progression through the MDA sequence. The investigation of the functionality of these miRNAs has uncovered a number of potential contributors to the underlying molecular mechanisms of BO and OAC development (Smith et al., 2010). In relation to this current study, HNF1 homeobox α (HNF1α), a transcription factor with a known relationship to CDX2, has been implicated in the regulation of miR194 (Hino et al., 2008, Benoit et al., 2010). Both HNF1α and miR194 have been demonstrated to have increased expression during the development of BO and OAC. A specific role for miR194 in the development of BO or OAC has not yet been identified (Wijnhoven et al., 2010). Further miRNAs have been identified as having significantly altered gene expression profiles during oesophageal carcinogenesis. While miRNAs and their role in oesophageal cancer were not investigated in this study, recent evidence has clearly indicated that they have an integral role to play in oesophageal disease progression.

In relation to the other potentially novel therapeutic targets and strategies that have been identified by this study, preventing the suppression of CDX2 as OAC develops from BO may be of importance to prevent the development of BO associated OAC. The data presented here are extremely preliminary and requires a substantial amount of validation and the execution of an siRNA library screen to identify potential modulators of this process. This avenue of research is however extremely promising. GATA6 has also been identified as a potential target to reduce the cell viability of dysplastic oesophageal cells. GATA6 is included in the druggable genome which is a collection of genes that are considered potential targets for therapeutics (Hopkins and Groom, 2002). As a potential therapeutic strategy using RNAi to target GATA6, the development stage is similar to any other target of RNAi which are all reliant on substantial progress with delivery systems. In relation to targeting GATA6 with a chemical or small molecule inhibitor, determining the molecular conformation of the protein using crystallography methods would aid in the development of a functional inhibitor. As GATA6 is a transcription factor, disruption of binding sites may be a viable approach to suppress GATA6 activity as a therapeutic approach.

7.1 Translational benefits of this study

The main aim of this study was to identify novel therapeutic targets for the treatment of oesophageal malignancies. The study has succeeded in identifying a number of potential therapeutic strategies and has also developed a robust system for the continued search of further therapeutic options. As described, there are a number of issues currently outstanding with respect to the use of RNAi as a therapeutic tool, however the technology associated with RNAi in the context of laboratory based research is well established. As such, RNAi screening is an invaluable tool in the discovery of novel therapeutic targets. The availability of siRNA libraries targeting subsets of the genome such as the druggable

genome facilitates a more rapid transition from a successful experiment in the laboratory to the integration of a new treatment option for the disease. Many targets of siRNA libraries currently have approved therapeutic options for use in patients with diseases unrelated to oesophageal malignancies such as small molecule inhibitors. Studies such as this have the potential to identify novel links between current therapeutic drugs and modulators of oesophageal malignancies. An siRNA library screen, utilising the protocols and systems developed in this study, has the potential to discover a new application for previously developed therapies, as well as identifying a number of RNAi targets which can be developed in tandem with RNAi delivery systems. Further projects expanding on and utilising the systems developed in this study will identify a novel therapeutic strategy to treat oesophageal adenocarcinomas and its pre-malignant stages.

7.2 Intellectual property

There are a number of potential patenting opportunities arising from this study with regards to the use of siRNA as a therapeutic strategy in the treatment of oesophageal cancer. A brief patent search revealed a precedent whereby a therapeutic strategy was developed and the patent granted for the treatment of influenza using siRNA technology (Eisen *et al.*, 2003). Current patent applications regarding the use of siRNA are pending and if granted may detract from applications generated from this study (Fire *et al.*, 2010). These applications will be monitored and a more comprehensive patent search will be undertaken by the technology transfer department of the university. The potential novel therapeutic targets that have been identified in this thesis for the treatment of oesophageal cancer include: the combined targeting of BCL-XL, MCL1 and GATA6 to reduce the proliferative potential of dysplastic oesophageal cells; inhibition of BCL-XL alone to resensitise oesophageal dysplastic cells to DCA induced cell death and also other

modulators of resistance to DCA induced cell death which have been identified such as FZD molecules.

7.3 Research studies in the societal context

Oesophageal cancer is an aggressive disease and if diagnosed at a late stage has an extremely poor prognosis. The development of alternative therapeutic options is imperative to decrease the potency of this disease. The numbers of cases of this cancer has been increasing over the last number of decades and as such will become better recognised and topical in the future. This study gives a solid foundation for the development of improved resources for the development of therapeutic options for this disease and to combat the growing impact that this cancer will have on society if improvements in treatment are not achieved.

7.4 Concluding remarks

In conclusion, this study has utilised functional siRNA screening to identify modulators of resistance to DCA induced cell death in oesophageal dysplastic cells. Further siRNA screens to investigate the cell proliferation of dysplastic oesophageal cells and to identify suppressors of CDX2 expression in the development of OAC have been developed. The preliminary investigations for both these screens searching for positive controls have themselves revealed a number of potential novel therapeutic strategies. Future studies will involve a series of validation experiments on the work that has been completed in this study. As well as discovering a number of positive candidates for the development of novel therapeutic strategies, this study has established an effective screening system that could be instrumental in the identification of further novel therapeutic strategies for the treatment of oesophageal malignancies.

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Strictly Private and Confidential

Ms. Fiona Behan
Abbeylands
Castledermot
Co Kildare

28 February 2011

Dear Ms. Behan,

Thank you for your letter concerning having a 'stay' of 5 years put on the Ph.D. thesis entitled "Bile acids and Barrett's oesophagus: The Development of a functional siRNA screen to identify novel therapeutic targets". I agree to this 'stay' being imposed.

When the hardbound copy of the thesis is being submitted to this office please ensure that you enclose a copy of this letter as it is at that point we will write to the Library advising them of the 'stay' and giving them the title of the thesis.

Yours sincerely,

Professor Veronica Campbell
Dean of Graduate Studies

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