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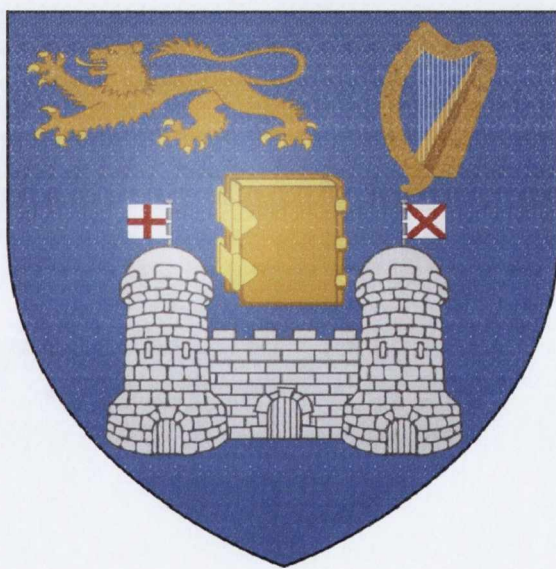
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**Regulators of Inflammation and Innate Immunity
Identified in Oesophageal Cancer Cell Viability
Through Implementation of a Druggable Genome
siRNA Library Screen**

Catherine E Garry MSc

**A Thesis Presented to the University of Dublin for the
Degree of Doctor of Philosophy**

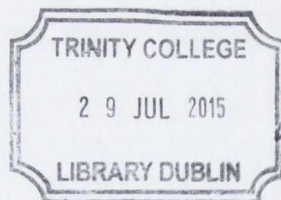


**PhD in Clinical Medicine
Supervisor: Prof. Dermot Kelleher**

2015

Declaration

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Thesis 10678

Summary:

Inflammation initiated by gastroesophageal reflux disease (GORD) can lead to development of oesophageal adenocarcinoma (OAC), through a premalignant lesion, Barrett's oesophagus (BO). OAC has a 5-year survival of patients with localised OAC of 38%, which decreases dramatically if the tumour has spread. Due to the heterogeneity observed within cancers, identification of successful therapeutics has proved difficult. Small interfering RNAs (siRNA) may be targeted to induce gene silencing and subsequent loss of protein expression and function. Commercially available siRNA libraries enable simultaneous analysis of the effect of thousands of genes on a particular outcome. This study aimed to define therapeutic targets for OAC by utilising a siRNA library screening with the intention of identifying novel therapeutic targets for the treatment of OAC.

Methodological and technical approaches necessary for a siRNA library screen were extensively optimised. Statistical approaches were tested using pilot siRNA library screening data to determine appropriate metrics and statistical interpretations for data analysis. The combination of these statistical tests for quality control (QC) was determined as an optimal strategy to identify siRNA gene targets which affect OAC cell survival. A druggable genome siRNA library screen was performed using high-grade dysplastic oesophageal cells. Statistical analysis using the quality control metrics; t-test, Z-factor and SSMD concluded the success of the screen. Hit selection analysis identified 118 high confidence hits that significantly reduce cell viability. Bio-informatical examination of the hit lists defined a large proportion of immune related gene targets, 12 of which were subsequently verified in cell lines spanning the

oesophageal carcinogenic sequence. Use of cancer gene expression databases indicated that, of these, LIF and C1QA may be dysregulated in OAC tissues.

Leukaemia Inhibitory Factor (LIF) is a secreted cytokine member of the IL-6 family, which binds to its receptors LIFR and gp130, resulting in STAT3 activation. Validation of the role of LIF, via siRNA mediated gene inhibition, showed a reduction in both cell survival and STAT3 activation in high grade dysplastic oesophageal cells. Thus LIF may be essential to the observed sustained STAT3 activation in OAC cell lines. Inhibition of LIF also resulted in a significant decrease in IL-6 mRNA expression suggesting that LIF may mediate IL-6 levels. C1QA is a member of the serum complement system, essential in innate immunity. Inhibition of C1QA significantly reduced high grade dysplastic cell survival with a further decrease noted upon addition of C1q from human serum, indicating a possible negative feedback loop. Inhibition of C1QA significantly increased β -catenin stabilisation and Wnt signalling, significantly reducing cell survival. A double knockdown was also performed between a panel of immune related genes, and either LIF or C1QA, identifying 3 genes RUNX2, TREM2 and PTCRA, which are all located within the same chromosomal region, 6p21. Variants within this region have previously been highlighted in genome wide association studies in association with BO.

Overall, this thesis demonstrates a novel method through which OAC cells can induce cell viability by utilising locally derived and innate immune factors expressed within the epithelial tumours themselves, identified through the implementation of a successful siRNA library screen of the human druggable genome. Thus, targeting these pathways may provide a novel approach for OAC specific future therapeutics.

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Abbreviations:

A549	Human Lung Adenocarcinoma Cell Line
AAMR	Acute Antibody-Mediated Rejection
ACP5	Acid Phosphatase 5
AEO	Allergic Eosinophilic Oesophagitis
APS	Ammonium Persulphate
BAC	Bacterial Artificial Chromosome
BCA	Bicinchoninic Acid
BCL-9	B-cell CLL/Lymphoma 9
BCL-xL	B-cell Lymphoma-Extra Large
BLAST	Basic Local Alignment Search Tool
BMP	Bone Morphogenic Protein
BO	Barrett's Oesophagus
BRCA1	Breast Cancer 1
BRCA2	Breast Cancer 2
BSA	Bovine Serum Albumin
C13K	Ovarian Cisplatin Resistant Cell Line
C1INH	C1 Inhibitor
C1q	Complement component 1, subcomponent Q
C1QA	Complement component 1, subcomponent Q, chain A
C1r	Complement component 1, subcomponent R
C1s	Complement component 1, subcomponent S
C8A	Complement Component 8, alpha polypeptide
CAC	Colitis-Associated Cancer
CASP10	Caspase 10
CER I	Cytoplasmic Extraction Reagent 1
CER II	Cytoplasmic Extraction Reagent 2
c-MYC	v-myc Avian Myelocytomatosis Viral Oncogene Homolog
CRC	Colorectal Cancer
CRP	C-Reactive Protein
CT-1	Cardiotrophin 1
CTCF	CCCTC-Binding Factor
CV	Cell Viability
DAVID	Database for Annotation, Visualisation and Integrated Discovery
DCA	Deoxycholic Acid
DCL	Dicer-Like
DMSO	Dimethyl Sulphoxide
DSA	Donor Specific Antibody
DUSP21	Dual Specific Phosphatase 21
Dvl	Dishevelled
EDTA	Ethylenediaminetetraacetic acid
EGFR	Epidermal Growth Factor Receptor
ELISA	Enzyme-Linked Immunosorbent Assay

EPC-2	Primary Human Oesophageal Keratinocytes
F8	Coagulation Factor VIII, Procoagulant Component
FBS	Foetal Bovine Serum
Fz	Frizzled
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA6	GATA Binding Factor 6
GEJ	Gastroesophageal Junction
GFP	Green Fluorescent Protein
GOhTRT	High Grade Dysplastic Cell Lines
GORD	Gastroesophageal Reflux Disease
gp130	Glycoprotein 130
GPCR	G-protein Coupled Receptor
H1703	Non-Small Cell Lung Cancer Cell Line
H520	Lung Squamous Cell Carcinoma Cell Line
HaCaT	Immortal Keratinocyte Human Cell Line
HAE	Hereditary Angioedema
HAR	Hyper Acute Reaction
HAT	Histone Acetyltransferase
HBSS	Hanks Balanced Salt Solution
HCH	High Confidence Hit
Hek293T	Transformed Human Embryonic Kidney 293 cells
HepG2	Hepatocellular Carcinoma Cell Line
Het1A	Normal Oesophageal Cell Line
HGD	High Grade Dysplastic
HIF-1	Hypoxia Inducible Factor 1
HIPK2	Homeodomain Interacting Protein Kinase 2
HLA	Human Leukocyte Antigen
ICAM1	Intercellular Adhesion Molecule 1
IHC	Immunohistochemical
IKBKB	Inhibitor Of Kappa Light Polypeptide Gene Enhancer In B-Cells, Kinase Beta
IL-6	Interleukin 6
ILF3	Interleukin Factor 3
IPA	Ingenuity Pathway Analysis
JAK	Janus Kinase
KAT8	K(Lysine) Acetyltransferase 8
KCNQ2	Potassium Voltage-Gated Channel, KQT-like Subfamily, Member 2
KRT10	Keratin 10
LB	Lysogeny Broth
LEF-1	Lymphoid Enhancer-Binding Factor 1
LGD	Low Grade Dysplastic
LIF	Leukaemia Inhibitory Factor
LIFR	Leukaemia Inhibitory Factor Receptor
LRH-1	Liver Receptor Homolog 1
LRP6	Low Density Lipoprotein Receptor-Related Protein 6

MCF-10A	Fibrocystic Mammary Gland Cell Line
MCL-1	Myeloid Cell Leukaemia Sequence 1
MED19	Mediator Complex Subunit 19
MHC	Major Histocompatibility Complex
MIO	Minimally Invasive Oesophagectomy
MNDA	Myeloid Cell Nuclear Differentiation Antigen 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide Cell Viability Assay
MTT	Assay
MXD1	Mas Dimerisation Protein 1
MYLK	Myosin Light Chain Kinase
NE-PER	Nuclear Extraction Protein Extraction Kit
NER	Nuclear Extraction Reagent
NFκB	Nuclear Factor kappa-light-chain-Enhancer of Activated B cells
NOD2	Nucleotide-Binding Oligomerization Domain-Containing Protein 2
NRIH2	Nuclear Receptor Subfamily 1, Group H, Member 2
NSAID	Non-Steroidal Anti Inflammatory Drug
OAC	Oesophageal Adenocarcinoma
OE33	Oesophageal Adenocarcinoma Cell Line
OR	Odds Ratio
ORF	Open Reading Frame
OSCC	Oesophageal Squamous Cell Carcinoma
OSM	Oncostatin M
OTE	Off-Target Effect
PBL	Passive Lysis Buffer
PFA	Paraformaldehyde
PGC-1α	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1-alpha
PLK1	Polo-Like Kinase 1
PPI	Proton Pump Inhibitor
PRM	Pattern Recognition Molecule
PRMT1	Protein Arginine N-methyltransferase 1
PSG	Penicillin/Streptomycin/Glutamine
p-STAT3	Phosphorylated Signal Transducers and Activator of Transcription 3
PTCRA	Pre T-cell Antigen Receptor Alpha
PTEN	Phosphatase and Tensin Homolog
PTTG	Pituitary Tumour Transforming Gene
PVDF	Polyvinylidene fluoride
QA/QC	Quality Assurance/Quality Control
R	R Software Package
RAN	Ras-Related Nuclear Protein
RELA	v-rel Avian Reticuloendotheliosis Viral Oncogene Homolog A
RISC	RNA-induced silencing complex
RRM2	Ribonucleotide Reductase 2
RUNX2	Runt-Related Transcription Factor 2

SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SH2	Src Homology 2
SIK2	Salt-Inducible Kinase 2
siRNA	Small Interfering Ribonucleic Acid
SK-GT-4	Oesophageal Adenocarcinoma Cell Line
SLE	Systemic Lupus Erythematosus
SON	SON DNA Binding Protein
SSMD	Strictly Standardised Mean Difference
STAT	Signal Transducers and Activator of Transcription
SYMPK	Symplekin
T24	Urinary Bladder Transitional Cell Carcinoma Cell Line
TCF	Transcription Factor
TEMED	Tetramethylethylenediamine
TGF-β	Transforming Growth Factor Beta
TLR	Toll-Like Receptor
TNBC	Triple Negative Breast cancer
TP53	Tumour Protein p53
TRAF2	TNF Receptor-Associated Factor 2
TREM2	Triggering Receptor Expressed on Myeloid Cells 2
TRPC7	Transient Receptor Potential Cation Channel, Subfamily C, Member 7
TRPV2	Transient Receptor Potential Cation Channel, Subfamily V, Member 2
UBB	Ubiquitin B
UTR	Untranslated Region
WB	Western Blot
Wnt	Wingless
WR	Working Reagent

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Chapter 1

General Introduction

1 General Introduction

1.1 Overview

There are two main types of oesophageal cancer; oesophageal squamous cell carcinoma (OSCC) and oesophageal adenocarcinoma (OAC), and while incidence of OSCC has shown a steady decline, the opposite has been found with OAC. The incidence rates of OAC vary between countries and ethnicities; in Western countries the incidence has increased significantly since the 1970's, with Scottish males showing the highest increase in incidence in Europe of 3.9% between the period 1978 to 1995 (Vizcaino et al., 2002), while in the USA an increase of 600% was noted in the past 40 years (Yang et al., 2012, Pohl and Welch, 2005). In 2010, there were 8,477 cases of oesophageal cancer diagnosed in the UK, with 7,610 deaths due to the disease (Cancer Research UK). Currently, Irish males have the third highest incidence rates in Europe (Figure 1.1), following only the UK and Holland, whereas Irish and UK females have the highest incidence rates of all EU countries. The major risk factors associated with OAC are gastroesophageal reflux disease (GORD) and Barrett's Oesophagus (BO), while environmental factors such as obesity and smoking can also increase risk of OAC onset (Hardikar et al., 2013, Lepage et al., 2013, Zhang et al., 2009a), all of which are discussed in more detail throughout this chapter.

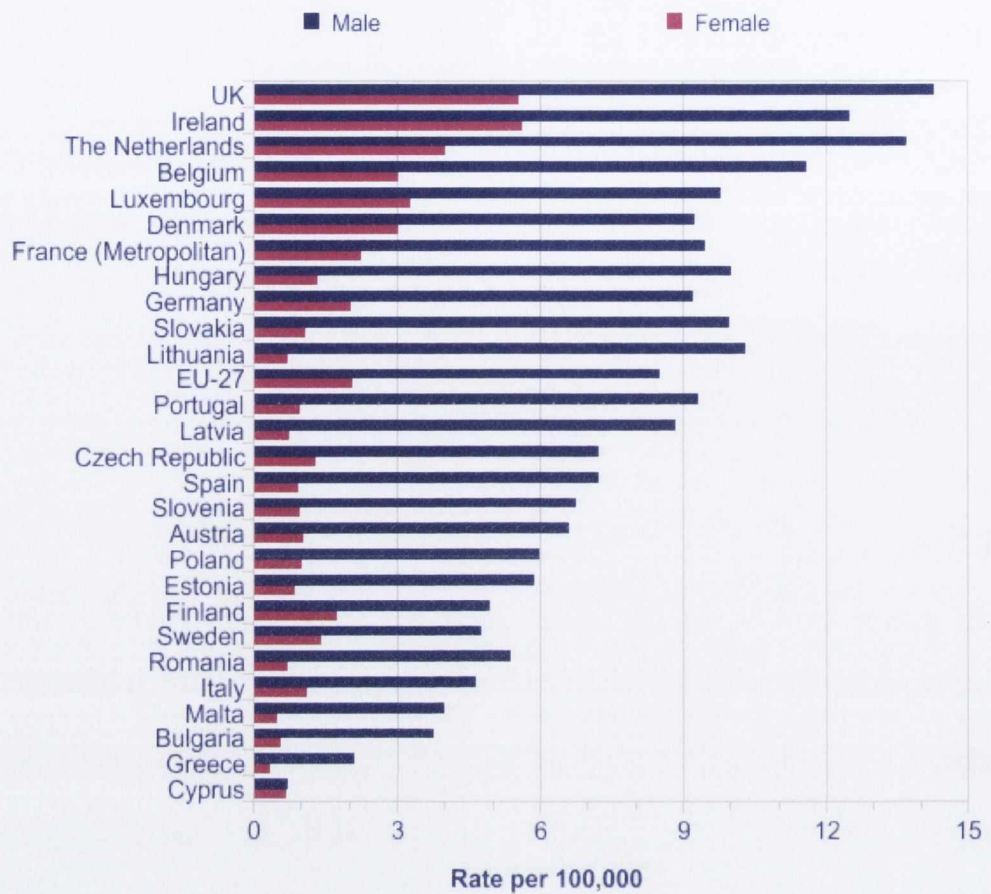


Figure 1.1: European Age-Standardised Incidence Rates per 100,000, EU-27 Countries, 2008 Estimates. UK has the highest male incidence of oesophageal cancer, while UK and Ireland have highest female incidence in Europe (*Cancer Research UK, 2013*)

1.2 Oesophageal Carcinogenic Sequence

The carcinogenic sequence from metaplasia to dysplasia and carcinogenesis is characterised by several features; an increase in cell proliferation, a loss of differentiation, reduced apoptosis and the expression of both growth factors and adhesion molecules, all of which are included in the hallmarks of cancer. The first six were elucidated in 2000 by (Hanahan and Weinberg, 2000) while it has also long been

suggested that there is also a clinical and epidemiological link between inflammation and cancer, leading to it recently been upgraded to one of the hallmarks of cancer (Figure 1.2). These phenotypic alterations that cancer cells undergo give them a distinct selective growth advantage over normal cells; therefore, the seven identified hallmarks are all biological processes which are acquired during tumour development.

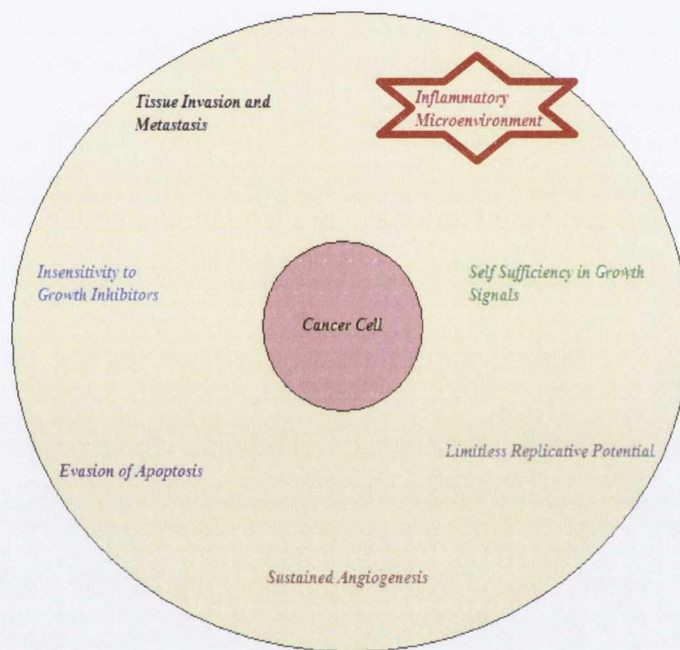


Figure 1.2: Hallmarks of Cancer. Each of these seven hallmarks of cancer, contribute to the development of cancer. The inflammatory microenvironment (highlighted with red star) has been a recent addition to the other six already established hallmarks.

Metaplasia is defined as the process in which a fully differentiated cell type replaces another (Spechler, 2013) while dysplasia is the explicit neoplastic alteration of the epithelium which is confined to the basement membrane of the gland where it first arose (Riddell et al., 1983). There are a number of reasons why such dysplastic changes can occur, frequently involving damage to the mucosa, which can cause both

genetic and/or epigenetic changes within the cells lining the oesophagus. The incidence of intestinal metaplasia and BO has been reported to increase the risk of developing OAC by as much as 30 to 125 times, compared to an age-matched population (Yousef et al., 2008). This highlights the need for a better understanding of these early stages within the carcinogenic sequence, as determination of the stage at which the patient is within the oesophageal carcinogenic sequence is currently the best available determinant of prognosis.

1.2.1 Genetic Abnormalities Within the Carcinogenic Sequence

Progression through the oesophageal carcinogenic sequence is characterised by a number of genetic and epigenetic abnormalities as outlined in Figure 1.3, including increased cyclin D1 expression, increased COX-2 activation and abnormal β -catenin localisation (nuclear). For example, cyclin D1 expression has long been associated with the progression from metaplasia to dysplasia, and can also stimulate cell proliferation of oesophageal squamous cells. It has also been demonstrated that Wnt activation and cyclin D1 are both overexpressed in OSCC, as cyclin D1 is a known target gene of the Wnt pathway. Interestingly, cyclin D1 levels are not reduced upon Wnt inhibition suggesting that there may be alternate regulating mechanisms behind cyclin D1 overexpression (Naganuma et al., 2012), which requires further investigation. COX-2 is also known to be activated in several malignant tumours including breast, resulting in a poor prognosis (Ristimäki et al., 2002). In OSCC high levels were found in dysplastic and invasive OSCC tissues as compared to normal samples (Zhi et al., 2006), with results indicating that COX-2 activation occurs early in the development and progression of OAC. By increasing our understanding of the molecular events involved in OAC development it is believed that major advances

could be made in the treatment of oesophageal disease. This may then allow for the development of novel therapeutic targets which could interfere in the early stages of tumour development thus preventing formation of an advanced tumour.

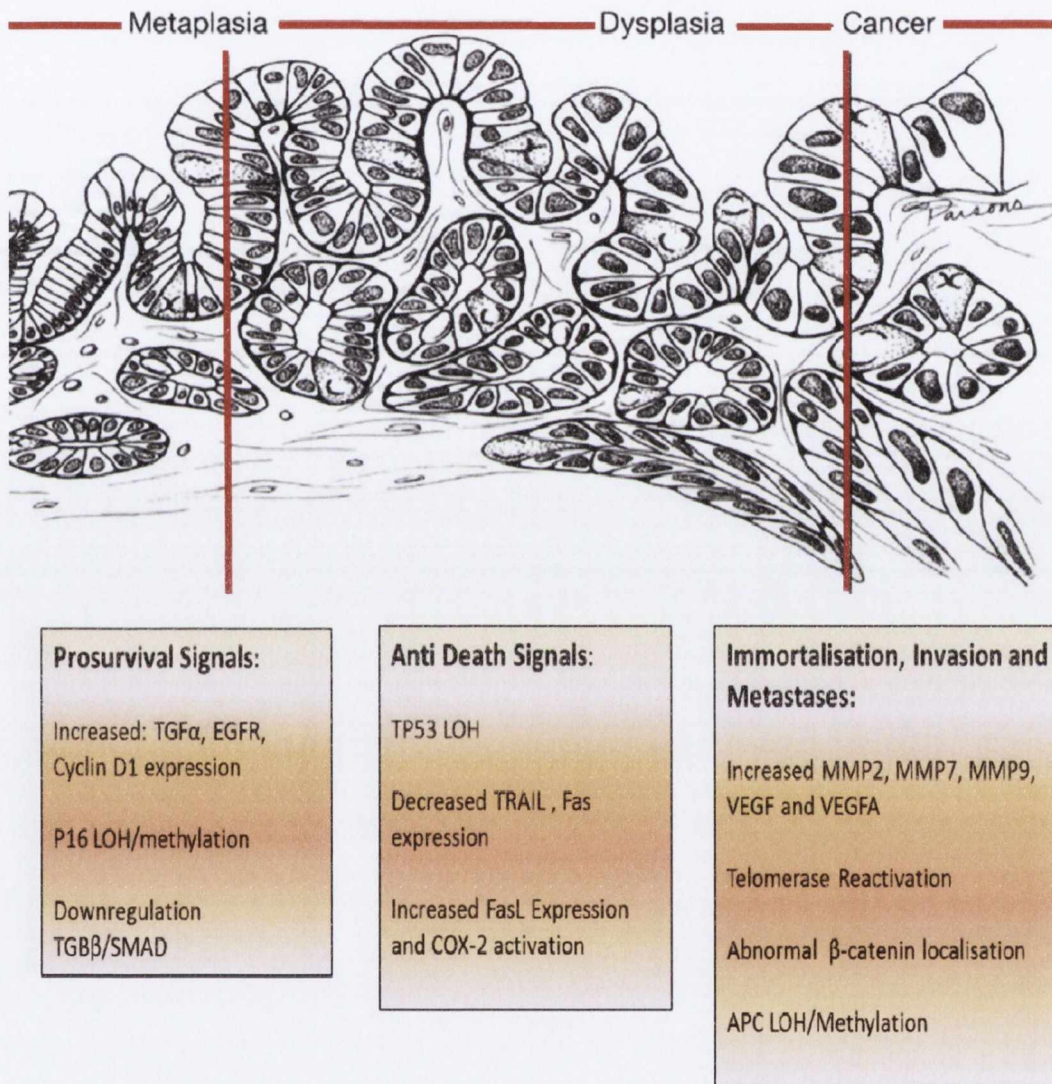


Figure 1.3: Genetic Events Occurring During the Oesophageal Carcinogenic Sequence. The oesophageal carcinogenic sequence is well characterised in which normal squamous epithelium transforms into oesophageal adenocarcinoma. Several important genetic alterations are indicated which are known to occur during progression through this sequence. (Modified from (Buttar and Wang, 2004).

1.2.2 Reflux Oesophagitis and Gastro-Oesophageal Reflux Disease

Chronic gastro-oesophageal reflux disease (GORD) is a major risk factor of BO that is characterised by the entry of gastric juices and bile salts (refluxate) into the distal oesophagus following a shortening or loss of function of the lower oesophageal sphincter (LOS). Repeated incidents of GORD can damage the mucosal layer which may lead to inflammation and ulceration of the oesophagus (Spechler, 2013), while healing of the injury caused by the refluxate often involves a metaplastic process whereby intestinal-type columnar cells replace the normal squamous epithelium (Spechler, 2013). There are several risk factors associated with development of GORD including presence of a hiatus hernia, *H. pylori* infection, smoking and obesity, resulting in symptoms such as heartburn and regurgitation which are characteristic of GORD.

1.2.2.1 Risk Factors of Gastro-Oesophageal Reflux Disease

Hiatus Hernia occurs when the gastro-oesophageal junction (GEJ) is deviated proximally, meaning that the intrinsic sphincter is situated higher than the hiatus which is formed by the crural diaphragm. This results in a hernia, due to the proximal stomach now being situated within the chest, which is thought to occur due to a weakening of the phreno-oesophageal ligament which joins the GEJ and the crura (Lee and McColl, 2013). Hiatus hernia can cause GORD by a number of methods including; acid being trapped within the hernia, impaired ability to clear the oesophagus and reduced functionality of the sphincter allowing for easier passage of the refluxate. The gastric cardia region of the stomach becomes more acidic after a meal as compared to the rest of the stomach and it is the acid from this region, known

as the acid pocket, that is contained within the oesophageal refluxate after meals (Clarke et al., 2009), which is larger in patients with hiatus hernia than those without. It is hypothesised that on ingestion of food, acid is secreted by the mucosa lining the hernia sac when there is no food to buffer the pH level resulting in more severe incidences of reflux due to the large amount of unbuffered acid present when the intrinsic sphincter does not work correctly (McColl et al., 2010).

In 2004, *Helicobacter pylori* was upgraded to a group I carcinogen for adenocarcinoma of the distal stomach by the World Health Organisation's (WHO) International Agency for Research on Cancer. It is a known cause of gastritis and can lead to an increased risk of peptic ulcers and gastric cancer (Cavaleiro-Pinto et al., 2011). It has been hypothesised that it is a risk factor for GORD, but this is controversial as no definitive evidence has thus far been identified. For example, in 2012, a study demonstrated that *H. pylori* was inversely associated with asymptomatic erosive oesophagitis (AEO), which confirmed previous experiments indicating that an inverse relationship was noted between *H. pylori*, BO and OAC (Wang et al., 2012a, Rokkas et al., 2007). These results indicate that *H. pylori* has a protective effect against development of BO and OAC which may be due to the fact that infections caused by *H. pylori* reduce the amount of gastric acid secreted therefore preventing GORD development (Lee and McColl, 2013, Zhang et al., 2009a). On the other hand, in a post hoc analysis of eight double-blind prospective trials of *H. pylori* therapy, it was shown that eradication in patients with duodenal ulcer disease did not result in development of erosive esophagitis, development of new symptomatic GORD, or worsening of symptoms in patients with pre-existing GORD (Laine and Sugg, 2002).

1.2.2.1.1 The Role of Lifestyle in Development of GORD and OAC

Environmental factors can also increase the risk of OAC development. For example, smoking has been identified as a risk factor for a number of cancers including lung, oesophagus and stomach (Bodelon et al., 2013, Cavaleiro-Pinto et al., 2011, Spechler, 2013). Not only does tobacco have a high carcinogen content, but it can also cause cancer development by triggering chronic inflammation (Takahashi et al., 2010). It has been demonstrated that smoking can increase the risk of progression from non-dysplastic BO to high-grade dysplastic BO by between 2- to 4-times that of non-smokers (Hardikar et al., 2013, Coleman et al., 2012), but although smoking is a risk factor there is no evidence thus far indicating that cessation of smoking can reduce reflux symptoms (Bredenoord et al., 2013).

Another environmental risk factor, obesity and associated reduced intake of fruit and vegetables, has been associated with a number of diseases including heart disease and cancer (Steevens et al., 2011). In 2009, the World Cancer Research Fund reported that 35% of oesophageal cancers are attributed to obesity (Research, 2009), which further supported previous meta-analysis findings that there was a positive association between increased BMI and OAC (Kubo and Corley, 2006). Research has shown that a BMI ≥ 40 has an odds ratio (OR) of 4.76 (95%, CI: 2.96–7.66) associated with OAC (males & females), and OR of 3.07 (95%, CI: 1.89–4.99) associated with adenocarcinoma of the gastro-oesophageal junction (GEJ) (males & females) (Hoyo et al., 2012) which correlates with a previous study indicating that the association between OAC and BMI ≥ 25 had an OR of 2.2 (95%, CI: 1.7-2.7) in males and OR of 2.0 (95%; CI 1.4-2.9) in females (Kubo and Corley, 2006). In the first published Irish study on the association between obesity and OAC, it was shown that the OR of

OAC in the lower oesophagus was 11.3 times higher (95%, CI: 3.5-36.4) in patients (male & female) who had a BMI >30 kg/m² as compared to patients who had a BMI <22 kg/m² (p<0.001) (Ryan et al., 2006). This study also showed that in males alone, the risk of adenocarcinoma of the GEJ was three-fold higher for males in the top quartile (OR of 2.9 (95%, CI: 1.4–6.1)) as compared to males in the lowest quartile (p<0.001).

The mechanisms through which obesity and increased BMI can lead to increased OAC risk remain generally unknown, but it is believed that they are likely to be multifactorial. One possible reason that central obesity may act as a risk factor may be due to the increased intra-abdominal pressure which increases the gastro-oesophageal pressure gradient (Zhang et al., 2009a, Lee and McColl, 2013, Pandolfino et al., 2006). This increase in pressure can predispose to hiatal hernia by pushing the GEJ towards the chest, although it has only been shown to be weakly associated, thus there must be a more plausible explanation for the observed association. Another possible mechanism may be the activation of pro-inflammatory pathways, through BO, which is known to be a precursor lesion which can predispose a patient to OAC. One of the major inflammatory pathways identified in BO is the IL-6/STAT3 pathway, of which the cytokine IL-6 plays an important role (Dvorak et al., 2007, Berishaj et al., 2007, Jarnicki et al., 2010). IL-6 is also secreted by metabolically active fat cell, along with other widely studied cytokines including TNF- α and IL-8 (Guzik et al., 2006). A feedback loop is thus created as adipocyte hypoxia can result in adipocyte tissue macrophage (ATM) infiltration and activation, recruitment of inflammatory cytokines into adipose tissue, resulting in a pro-inflammatory state, thus expressing further

cytokines (IL-6 and TNF-a), and releasing them into the portal and systemic circulation (van Kruijsdijk et al., 2009, Ryan et al., 2011).

Lack of exercise can also be a risk factor for developing OAC, as a one large scale study of both men and women in the USA identified that increased physical activity was associated with a decreased risk of adenocarcinomas of the upper gastrointestinal tract (Leitzmann et al., 2009). The study also suggested that some exercise is better than none at all, as the apparent protective effect was reached at a relatively low threshold. In a more recent meta-analysis, it was shown that oesophageal cancer risk was 29% lower among the most physically active compared to the least physically active subjects (OR, 0.71; 95% CI, 0.57-0.89) (Singh et al., 2014). This study also identified that physical activity was decreased the risk of developing OAC by 32% (4 studies, 503 cases of EAC; OR, 0.68; 95% CI, 0.55-0.85). In relation to OSCC, there were only 3 studies, and there did not appear to be any association noted (OR, 1.10; 95% CI, 0.21-5.64). The general conclusion that can be drawn from these studies is that overweight and lack of exercise can increase risk of OAC development, particularly but not exclusively in white males, therefore controlling obesity rates and promoting a more healthy lifestyle could help reduce the risk of OAC development.

1.2.2.2 Acid Reflux and the Refluxate

Pathological abnormalities such as intestinal metaplasia of the gastric cardia, BO and inflammation can be caused by acid reflux (Lee and McColl, 2013) as previously mentioned. The composition of the refluxate (gastric contents) is quite complex, as it can also include contents from the duodenum such as bile acids and pancreatic juices, and air. A number of factors can help determine the contents of the refluxate

including; speed of gastric emptying, post-prandial mixing of the gastric contents, the presence of an acid pocket, and also the use of PPI's. Generally, the stomach contents are acidic with a pH of 1.5-3.5, although this may be higher in patients treated with PPI's, and the mixing of stomach acid with food can increase the pH, therefore reducing the risk of damage to the oesophageal lining. An acid pocket contains stomach contents that are not mixed with food so that the pH remains low, and when a reflux episode occurs it can cause mucosal damage; while in patients with GORD the presence of an acid pocket is common, this pocket is generally larger in patients with a hiatus hernia (McColl et al., 2010).

Not all patients with acid reflux present with symptoms of oesophagitis, suggesting a role for the other contents of the reflux. The refluxate consists of acid, gastric juice components such as pepsin, and elements of the duodeno-gastro-oesophageal reflux (DGOR) such as bile acid. Pepsin is a highly acidic protein that is secreted in the gastric juice and is activated by low pH levels. In 2001, Tobey et al., demonstrated that exposure of rabbit oesophageal tissue to acidic pepsin ($\text{pH} < 3$) increased tissue damage and produced an irreversible lesion, while a reversible lesion was shown after exposure to acid alone (Tobey et al., 2001). This irreversible lesion resulted in paracellular permeability, which may lead to development of erosive damage by allowing acids access to the basolateral membrane of the oesophageal cells.

Bile acids are organic anions that are the primary constituent of bile that aid fat digestion. Cholesterol metabolism results in the formation of cholic and chenodeoxycholic acids which are the primary bile acids, while the secondary bile acids; deoxycholic and lithocholic acids, are produced in the colon by bacterial

dehydroxylation of the primary bile acids and have potentially oncogenic effects (Pai et al., 2004, Baek et al., 2010). Several studies have highlighted the importance of bile reflux in development of Barrett's metaplasia. For example, neoplastic progression of Barrett's metaplasia has been highlighted in two patients that had bile reflux but not pathological acid reflux (Jankowski et al., 1993), and exposure of the oesophagus to total bile acids shows a correlation with worsening mucosal damage leading to BO (Dixon et al., 2001). Metaplasia can occur due to the presence of chronic inflammation caused by the refluxate, which may predispose to dysplasia and malignancy (Picardo et al., 2012), and in the oesophagus, chronic inflammation caused by GORD can result in intestinal metaplasia which appears to be more resistant to injury by the refluxate, resulting in BO (Zhang et al., 2009a).

1.3 Barrett's Oesophagus

Barrett's oesophagus (BO) is the replacement of normal stratified squamous epithelia with metaplastic columnar epithelium, which can be induced by gastroesophageal reflux disease (GORD) in the distal oesophagus (Picardo et al., 2012, Yang et al., 2012, Spechler, 2013, Jankowski et al., 2000). Damage to the normal epithelia, due to acid reflux, can result in inflammation and replacement of the injured cells by intestinal columnar cells rather than the original normal squamous cells. Intestinal type metaplasia results in the surface epithelia showing villous type edges (Figure 1.4), with intestinal-like crypts that are lined by columnar cells which secrete mucous, and goblet cells that contain mucin (Prasad et al., 2010). There are two other histological types of columnar epithelial metaplasia, gastric fundus and junctional types, but it is only the intestinal type that predisposes to adenocarcinoma (Lekakos et al., 2011).

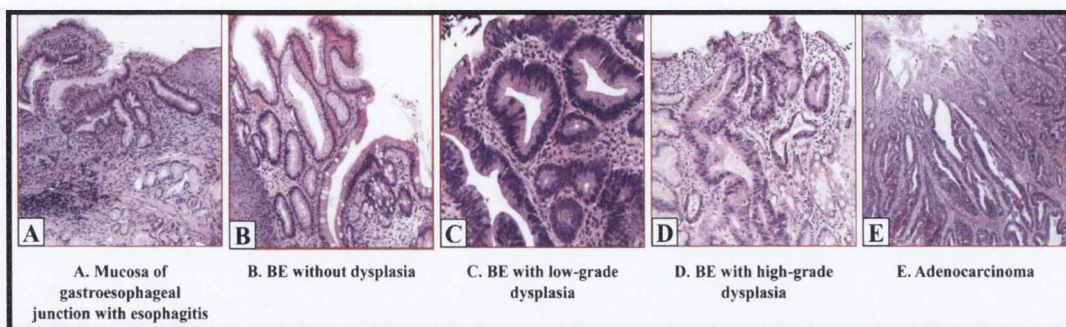


Figure 1.4: Progression from Normal Squamous Epithelium to Oesophageal Adenocarcinoma. Normal distal oesophageal squamous cells transform to adenocarcinoma via a number of steps including BO without dysplasia, low grade dysplasia (LGD) and high grade dysplasia (HGD) (*Conteduca et al., 2012*)

Although the rate of transformation from BO to OAC is variable, it is believed that from BO without dysplasia to OAC the rate is 0.5% per annum, while it can increase to up to 10% per annum in patients with low grade dysplasia (Lin et al., 2012, Jankowski et al., 2000, Conteduca et al., 2012), and for patients with high grade dysplasia the rate of transformation has been reported to be up to 20-40% (Collard, 2002, Rastogi et al., 2008, Reid et al., 2000). Dysplasia can occur in two grades with BO; low grade and high grade dysplasia, and is thus far the only widely used predictor for invasive adenocarcinoma development. Dysplasia can extend to either the basement membrane, into the lamina propria or muscularis mucosa, which is termed 'intramucosal adenocarcinoma', but if it penetrates further into the muscle tissue of the oesophagus it is referred to as 'invasive adenocarcinoma' (Lekakos et al., 2011). The latter is recognised at a much later stage of cancer development, which must therefore be treated more aggressively.

1.3.1 Proposed Theories for the Origins of Barrett's Oesophagus

BO develops when oesophageal squamous cells are replaced with intestinal metaplasia, although the exact mechanism by which this occurs remains unknown. There are four main theories that have come to light which may help to elucidate the origin. The first theory is that the BO type cells migrate upwards from the cells at the transitional zone of the gastroesophageal junction, thereby colonising the distal oesophagus or gastric cardia as a response to damage caused by the refluxate (Barbera and Fitzgerald, 2010, Hamilton and Yardley, 1977). If this theory were correct an uninterrupted progression of the cardia to BO mucosa would be present along the oesophagus, but this theory has not been supported by canine studies, in which it was shown that columnar epithelium can still be present above a squamous barrier (Li et al., 1994).

The second theory put forward is that BO originates due to transdifferentiation, which is the irreversible conversion from one fully differentiated state to another. This theory was supported by a murine study suggesting that squamous oesophageal cells can arise directly from columnar basal cells, without the need for cell division (Yu et al., 2005). In contrast, refuting this theory is the evidence that radiofrequency ablation of BO epithelium results in the development of new squamous epithelium (Chadwick et al., 2014).

A third theory suggests that stem cells may play a role in the development of BO. It is suggested that BO may develop from the change of multipotent stem cells, from normal squamous to columnar epithelial due to repeat exposure to an environmental stress such as the refluxate. There are two main types of stem cell; epithelial stem

cells, and stem cells that are non-intrinsic to the epithelium (gland duct theory). The epithelial stem cells located in the basal layer can give rise to the distinct cell lineages identified in adult tissue. It is thus postulated that these basal epithelial cells maintain the normal epidermis, and act to replace cell loss following injury as described in the epidermis (Clayton et al., 2007); investigations are now underway to determine if this theory holds true for the oesophageal epithelium. Recent evidence in mice has shown that the oesophageal epithelium is maintained and repaired by a single progenitor cell population which can switch between homeostatic and regenerative behaviour in response to injury (Doupe et al., 2012). Although this study did not find evidence of a slow-cycling epithelial cell as described by Clayton et al., it did identify that in response to injury by retinoic acid, epithelial progenitor cells can establish a homeostatic state for wound healing. The gland duct theory is based on the idea that stem cells may also be located in the glandular neck region of the oesophageal submucosal gland ducts. In 2008 a study indicated that normal squamous ducts contained the same somatic mutations as adjacent metaplastic epithelium, suggesting that Barrett's metaplasia was genetically heterogeneous and contained independent clones (Leedham et al., 2008). In contrast to this it was proposed that Barrett's metaplasia glands are clonal populations (Nicholson et al., 2012) indicating that within Barrett's glands was a population of cells that were deficient in cytochrome c oxidase (CCO), but which contained different cell lineages of BO, including goblet cells, neuroendocrine cells and foveolar cells, suggesting that the cells within the gland are multipotent stem cells. Intestinal metaplasia generally presents on the surface of the oesophagus with goblet cells and foveolar cells (mucus secreting) in between, while neuroendocrine cells (serotonin secreting) have also been identified within BO, and are generally indicative of intestinal epithelium (Lewin et al., 1992,

Koppert et al., 2004). This study by Nicholson et al., proposed that the heterogeneity identified by Leedham et al., may have been due to multiple rounds of division of progenitor glands (Nicholson et al., 2012). The stem cell theory could explain both the presence of various cellular phenotypes in BO and how the epithelium can repair itself following ablation of BO.

The fourth theory is the presence of a cell of origin for BO, as demonstrated by Wang et al. This paper suggested that BO is initiated from a cell of the embryo that persists at the oesophago-gastric junction and that the initial spread of this cell is derived from competitive interactions between cell lineages (Wang et al., 2011). This study used p63 null mice, in which the upper gut is lined with columnar epithelium rather than the normal squamous epithelium. The authors suggest that this is similar to human BO as IHC staining indicated the presence of keratin 8, Vilin and anterior gradient 2, which are all well-known markers for BO (Wang et al., 2009b, Pohler et al., 2004, van Baal et al., 2008). On further analysis of the source of epithelial metaplasia, common markers for BO were traced including Cldn3, Krt7, Krt8, Car4 and Muc4 which were found in a monolayer of cells lining the proximal stomach of embryonic mice. Interestingly, while Cldn3, Krt7, Krt8, and Muc4 signals became weaker as the embryo developed, the Car4 antibody signal was stronger and largely expressed on the monolayer of cells. On comparison of the Car4 staining pattern on the p63 null and wild type proximal stomach, both showed a similar pattern of Car4-expressing columnar epithelium. Given this, the authors suggest that the Car4-positive, columnar epithelium may develop into a metaplasia in the proximal stomach of the p63 null mice (Wang et al., 2011). A caveat associated with this study is the lack of Cdx2 expression in the p63 null mice the presence of which has been widely published in

BO (Clemons et al., 2013, Phillips et al., 2003). Cdx2 is a caudal-related homeobox family member and is known to play an important role in intestinal epithelial development and differentiation. The author's state that the lack of Cdx2 in the p63 null mice may suggest that transdifferentiation to an intestinal phenotype is probably not required for BO development. Furthermore, the architecture of murine oesophageal tissue differs from human as it lacks crypts and gland structures, therefore it is hard to exactly replicate the features of human BO in mice (Pavlov and Maley, 2010).

Metaplasia can occur within the body in the context of wound healing, which is characterised by inflammation, tissue formation and tissue remodelling (Slack and Tosh, 2001). In relation to the oesophagus, it is postulated that wound healing occurs due to mucosal injury caused by acid reflux. Wound healing is a complex process involving different cell types including keratinocytes, fibroblasts, endothelial cells, macrophages, and platelets, and it is the infiltration, proliferation, and differentiation of these cells that can induce the inflammatory response. Myofibroblasts have been shown to both synthesize extracellular matrix components including collagen types I and III, while they can also disappear by apoptosis when epithelialization occurs during normal wound healing (Gabbiani, 2003). Injured oesophageal epithelial cells can recruit inflammatory leukocytes which activate TGF- β inducing myofibroblast differentiation of the target cell at the site of injury. Intestinal myofibroblasts are crucial for the expression of the intestinal differentiation gene CDX2, which has been widely identified in BO (Duluc et al., 1997, Clemons et al., 2013, Phillips et al., 2003). Thus, it has been suggested that damage to the oesophageal epithelium by acid reflux, would lead to healing, and thus metaplasia, resulting in BO.

1.3.2 Treatment Options for Barrett's Oesophagus with Dysplasia

As previously mentioned, BO can occur as a consequence of bile acid reflux but it is essentially asymptomatic and normally only detected incidentally during an endoscopy, thus the true prevalence of BO is difficult to ascertain. If reflux symptoms are present, patients are generally treated with proton pump inhibitors (PPI) which inhibit the gastric acid. Although PPI's don't normally cause regression of metaplasia they can treat oesophageal reflux, but when treatment stops the symptoms will return. Besides medical treatment with PPI's, lifestyle and diet changes are recommended to reduce the effects of reflux; reduced fat intake, cessation of smoking, weight loss and elevation of the head of the bed to reduce night-time reflux episodes are all recommended although no evidence has proven that these methods impact on Barrett's metaplasia.

More invasive options for treatment of BO with dysplasia include radiofrequency ablation in which high frequency radio waves are administered to ablate the dysplastic mucosa with the intention of regrowth of normal squamous mucosa. This treatment has resulted in positive outcomes thus far, and is now a common treatment for dysplastic BO (Chadwick et al., 2014). Another option is an oesophagectomy which is normally recommended for patients with high grade dysplastic BO. This procedure is highly invasive and therefore has extremely high morbidity and mortality rates, ranging from 30%-80% for morbidity and 8%-23% for mortality (Chen et al., 2013a). The quality of life of patients may also be decreased following surgery, therefore given this, combined with the invasiveness of this surgery, the use of minimally invasive oesophagectomy (MIO) is now widespread, and can be used on both high grade dysplasia and localized tumours. There are several types of minimally invasive

surgery including laparoscopic and thoracoscopic surgery, both of which reduce the amount of pain and discomfort endured by the patient, while increasing the rate of recovery. Large scale studies are needed to confirm improvements in prognosis, but so far these techniques are gaining acceptability as compared to open surgery (Douard et al., 2004).

1.4 Oesophageal Cancer

Oesophageal squamous cell carcinoma (OSCC) occurs in the flat cells lining the oesophagus (Ortiz et al., 2010), while oesophageal adenocarcinoma (OAC) is the formation of a tumour within the sub-mucosal layer of the oesophagus (Prasad et al., 2010), and is now the predominant histological subtype in the Western world. BO-associated adenocarcinomas are generally located in the distal third of the oesophagus in areas which have previously been diagnosed with BO and frequently extend into the proximal stomach.

OAC cells are generally columnar in shape and they contain irregular nuclei with large amounts of euchromatin, prominent nucleoli, and varied amounts of clear cytoplasm. The tumour cells are generally arranged in sheets and poorly formed glandular lumina (Glickman, 2003). At the time of clinical presentation most OAC tumours have spread into or through the muscularis, while advanced tumours may have even spread directly into the mediastinum, aorta, or stomach and even metastasised to regional lymph. Tumours are classified by their size, from small to large; T1, T2, T3 and T4. Within each of these sizes, they are again subdivided by depth of invasion, for example in OAC the T1 classifications are; T1a, invading into lamina propria; T1b, into muscularis mucosae; T1c, into superficial submucosa; and

T1d, into deep submucosa. Location, size and depth of an early OAC tumour play a key role in its progression, for example, one study of 90 early oesophageal adenocarcinomas, showed that 0% of tumours limited to the mucosa (T1a) had lymph node metastases, compared with 36% of tumours in the submucosa (T1d) (Liu et al., 2005). In terms of prognosis, the larger the size and depth of invasion, the lower the rate of 5-year survival, with many studies indicating the 5-year survival for each stage; T1 survival is 81-100%, T2 survival is 24.1-51%, T3 survival is 14-15.1% and T4 survival is 0% (del Genio et al., 2006, Portale et al., 2006, Torres et al., 1999). Treatment options for patients diagnosed with OAC are limited and depend on the stage and location of the tumour, but eligible patients are treated with neo-adjuvant chemo-radiotherapy followed by surgical resection to remove the tumour.

Cancer research has mainly focused on the cancer cells themselves, but tumour cells don't exist in isolation, thus the role of the tumour microenvironment and the impact of cellular cross-talk are increasingly being investigated. The tumour cells themselves are reliant on their environment for both tumour development and progression (Allen and Louise Jones, 2011). Carcinoma-associated fibroblasts (CAFs), frequently identified in the stroma of human carcinomas, consist of both fibroblasts and myofibroblasts and due to their abundant presence, they are often associated with development of high-grade malignancies and subsequent poor prognoses (Shimoda et al., 2010). In normal tissue the stroma may act as a barrier preventing tumourigenesis by reducing cancer cell proliferation, but during tumourigenesis the stroma can evolve and change to aid tumour growth in response to molecular signals from carcinoma cells and other host cell types (Shimoda et al., 2010). Myofibroblasts are the main cell population found in CAFs and stromal fibroblasts that are exposed to carcinoma cell

medium, can differentiate into myofibroblasts, and have been identified as being better at promoting tumour growth (Guo et al., 2008, Noma et al., 2008). It is hypothesised that carcinoma cells can initiate the change of the stromal fibroblasts into myofibroblasts while also helping to maintain their activated phenotype *in vivo*.

The inflammatory response in the tumour microenvironment is characterised by infiltration of immune cells and chronic inflammation of the tissue can lead to tumour formation (Picardo et al., 2012). The presence of immune cells within tumours and the inflammatory environment can aid tumour development by firstly producing factors that support carcinogenesis and also by helping the tumour to evade the recognition by the host. Dendritic cells are increased in BO tissue as compared to normal oesophageal tissue and are often found clustered with T- and B-cells in the lamina propria (Bobryshev et al., 2009). Normally they help to initiate and maintain anti-tumour immunity, but within the tumour microenvironment they may be polarised into immunosuppressive regulatory dendritic cells which can induce tumour growth and progression by limiting the activity of effector T-cells (Ma et al., 2013). Precursor CD7+ T-cells are frequently identified in OAC tissue, while helper T-cells are found in both the normal squamous and also BO tissues (Berndt et al., 2010). NF- κ B activation (an inflammatory marker which will be discussed more throughout this thesis), apoptosis and caspase activity are increased in these T-cells in both BO and OAC, thus highlighting the role that the inflammatory infiltrate plays, and its importance in sustained inflammatory cytokine release. This increase in apoptosis thus suggests that they don't develop into mature effector T-cells, and that the immune response is compromised in malignant transformation (Picardo et al., 2012). It has also been identified that eosinophils can infiltrate the mucosa in a number of

BO patients, which is associated with basal cell hyperplasia (Ravi et al., 2011), while macrophages are generally increased in OAC as compared to BO, and they also produce vascular endothelial growth factor (VEGF) which promotes angiogenesis (Couvelard et al., 2000, McDonnell et al., 2003). Combined, these findings highlight the importance of the tumour microenvironment in relation to cell-to-cell signalling and the inflammatory infiltrate, which play a significant role in the development and progression of OAC.

1.4.1 Identification of the Hallmarks of Cancer Within Oesophageal Adenocarcinoma

The TP53 gene is one of the most important tumour suppressor genes, which acts as a potent transcription factor with a fundamental role in the maintenance of genetic stability. p53 is activated in response to cellular stress and DNA damage and it can trigger cell-cycle arrest, DNA repair and apoptosis, while it can also play a role in senescence, DNA metabolism, angiogenesis, cellular differentiation, and the immune response (Xu et al., 2008, Menendez et al., 2013, Yu et al., 2014). Germline mutations of p53 are also associated with Li-Fraumeni syndrome, a genetic condition in which patients present with multi-site tumours (Frebourg et al., 1995), while somatic mutations have been identified in several cancers by loss of heterozygosity (LOH) mutations, with over 50% of human tumours presenting with a loss of function mutation (Ozaki and Nakagawara, 2011). These mutations can result in mutant forms of TP53 which lack the sequence-specific transactivation ability resulting in impaired TP53 activity which promotes the accumulation of DNA damage in cells, leading to a cancer phenotype (Sakaguchi et al., 1998). Patients with LOH in TP53 are 16 times more likely to progress to OAC (Reid et al., 2001), while accumulations

of p53 protein were present in 52% of 137 patients following immunohistochemical (IHC) staining of resected OAC samples (Langer et al., 2006). TP53 alterations are involved in both early stage tumour development, and progression (Iwamatsu et al., 2001); therefore it is considered an important biomarker for diagnosis, tumour progression, and poor prognosis associated with lymph nodes metastasis.

Both Cox-1 and Cox-2 are important enzymes involved in both inflammation and tumour growth. Cox-1 is homeostatically constitutively expressed in a wide variety of tissues, while Cox-2 is permanently stimulated by a number of cytokines and growth factors and is usually present in inflamed tissue (Fu et al., 1999, Taddei et al., 2013). Overexpression of COX-2 is associated with decreased apoptosis, cell to cell adhesion, increased proliferation and angiogenesis (Mobius et al., 2005, Hashimoto, 2012), and has been associated with aggressive phenotypes in several cancer types including breast and colon cancers (Jana et al., 2012, Rahman et al., 2012). Within the oesophageal carcinogenic sequence, Cox-2 expression and cellular proliferation were also shown to be increased, from metaplasia to dysplasia and adenocarcinoma (Taddei et al., 2013).

This study also confirmed the involvement of COX-2 in the pathogenesis of the early histological modifications involved in reflux leading to OAC development. Upregulated COX-2 expression in cancer has been correlated with increased vascular endothelial growth factor (VEGF) production, a known potent regulator of angiogenesis as reviewed by (Toomey et al., 2009). Increased VEGF expression has previously been shown in BO, especially in the early stages of the BO sequence (Couvelard et al., 2000, Taddei et al., 2013, Mobius et al., 2003) and a recent study

showed that VEGF expression correlates with the stage of the disease, across the metaplasia-dysplasia-adenocarcinoma sequence. This study demonstrated that both the protein expression and VEGF production increased with disease progression, thus confirming the role of VEGF in angiogenesis within the oesophageal carcinogenic sequence, especially in the early stages (Taddei et al., 2013).

1.5 Highlighting the Link Between Immunity, Inflammation and Cancer

Inflammation is known to enhance both tumour initiation and progression, and can be triggered by either infection or tissue injury. There are several types of inflammation which can occur, which differ by cause, mechanism of action, intensity of inflammatory response, and outcome. Chronic inflammation in response to unresolved infection precedes tumour development in certain tumour types. For example, infections caused by *H. pylori* or hepatitis B have been associated with gastric cancer and hepatocellular carcinoma respectively (Cavaleiro-Pinto et al., 2011, Damania et al., 2014). The inflammatory response to environmental factors such as tobacco or asbestos is a different type of inflammation leading to the release of cytokines including IL-1 β , which may mediate enhanced cell growth or reduced apoptosis (Dostert et al., 2008). Another type of inflammation arises after tumour development which is caused by the tumour triggering an intrinsic inflammatory response resulting in a pro-tumorigenic microenvironment. This leads to recruitment of immune related leukocytes, increased expression of cytokines and chemokines, and induces angiogenesis (Newton and Dixit, 2012, O'Riordan et al., 2005, Mobius et al., 2003). This was first identified in 1863 by Rudolph Virchow who realised that many

malignancies were infiltrated with leukocytes indicating that malignant tumours arose at the site of chronic inflammation (Heidland et al., 2006).

1.5.1 The Innate Immune Response

There are two main types of normal immune response; innate and adaptive. The innate immune response is not specific to a particular pathogen therefore differing from adaptive immune response. It depends on a limited number of receptors and secreted proteins that are encoded in the germline that recognise conserved features or “patterns” of pathogens and is activated quickly to help destroy invaders. Innate effector cells act without antigen-specific receptors unlike T- and B-cells, and they also demonstrate a various range of functions such as limiting the expansion of microorganisms which include viruses, bacteria, and parasites. Various types of innate effector cells have been identified including; natural killer (NK) cells, LT α i cells which produce IL-17 and IL-22, and Th2-type innate lymphocytes such as natural helper (NH) cells and nuocytes (Koyasu and Moro, 2012).

1.5.1.1 The Role of Pattern Recognition Receptors in Innate Immune Response

It is essential for the innate immune response to keep the growth of invading microbes at bay until antigen-specific adaptive immune responses are established. The ability of the innate immune system to recognise and limit microbes early during infection is due to activation of complement, phagocytosis, autophagy, and immune activation by various pattern recognition receptors (PRR) families (Mogensen, 2009). PRRs such as the toll-like receptor (TLR) family are located on cell surfaces and can detect pattern-associated molecular patterns (PAMP). Examples of PAMPs include microbial nucleic acids, lipoproteins, and carbohydrates, or damage-associated molecular

patterns (DAMPs) released from injured cells (Newton and Dixit, 2012). These then form a complex resulting in the release of factors that recruit leukocytes to a specific region, including monocytes and neutrophils. The characterisation of human Toll analogue (TLR4) in 1997, has paved the way for studies demonstrating the importance of microbial pattern recognition in innate immunity, since then, ten TLRs have been identified in humans, each of which can recognise unique ligands and has distinct specificity for pathogen detection. TLR1, TLR2, TLR4, TLR5, and TLR6 occur on the surface of antigen-presenting cells (APCs) and recognise a range of pathogenic “patterns” including for example lipopolysaccharide and dimuramyl peptides. (Medzhitov et al., 1997, Mogensen, 2009). On the other hand, TLR3, TLR7, TLR8, and TLR9 act to recognise foreign nucleic acids. TLRs use shared adaptors including myeloid differentiating factor 88 (MyD88) and Mal/TIRAP, for signal transduction and downstream NF- κ B activation (Brown et al., 2011) with the exception of TLR3, which signals through TIR domain-containing adaptor inducing IFN-Beta (TRIF) (Hardy et al., 2004). Tumours show increased levels of various TLRs in several cancer types including oesophageal squamous cell carcinoma, and it has been demonstrated that mRNA and protein levels of TLR3, 4, 7 and 9, were increased significantly ($P < 0.05$) in OSCC tumours as compared to normal oesophageal tissue (Sheyhidin et al., 2011).

1.5.1.2 The Complement System and Innate Immune Response

The complement system plays a very important role in innate immunity, as it destroys an invading microorganism and removes immune complexes (Wang et al., 2012b), thus acting as an effector of innate immunity. It acts as a first defence against non-self

cells, and regulates several functions including; control of adaptive immunity, cell lysis and immunological and inflammatory processes (Ricklin et al., 2010). The presence of the complement system was demonstrated by Nobel prize winner, Jules Bordet, who found that if serum supplemented with an antibacterial antibody, heated to 37°C was added to a bacterium, it became lysed, whereas if the serum was heated to a temperature of 57°C then the bacterium were not lysed. The conclusions drawn from this were that the serum must then contain a heat-labile component that *complements* the lytic ability of the antibody (Wood, 2004).

1.5.1.2.1 Complement Pathways

There are three pathways in the complement system; classical, alternative and lectin pathways. These pathways differ in their mechanisms of target recognition but all three pathways activate the C3 component, which initiates C5 cleavage, resulting in membrane attack complex (MAC) activation (Figure 1.5). C1q, MBL and ficolins are pattern recognition molecules (PRM) that bind to the cell surface and initiate formation of C3 convertase of each pathway respectively resulting in C3 cleavage and formation of C5 convertase (Ricklin and Lambris, 2013a, Pio et al., 2013). The main step in complement activation is when the opsonin, C3b, generated during C3 cleavage, covalently binds to the microbial surface or to an antibody bound antigen, where it becomes a component of the enzyme which cleaves C5. The generation of C5a, during C5 cleavage, also contributes to inflammation (Wood, 2004), and it has been shown that binding of C5a to its receptor C5aR activates immune cells (Gunn et al., 2012).

1.5.1.2.2 The Role of Complement in Disease

Complement recognises non-self elements, and studies have shown that it can also recognise diseased and damaged host cells, therefore it is postulated that tumour cells should be recognised and targeted by complement. The balance of complement activation is fragile and if poorly regulated can potentially impact on diseases such as transplant rejection, systemic lupus erythematosus (SLE) and Alzheimer's disease (AD).

1.5.1.2.2.1 Transplant Rejection

Upon recognition of non-self surfaces, such as transplants, excessive complement activation has been identified, and can either be due to infection, ischemia-reperfusion or the formation of immune complexes such as xenoantibodies (Chen et al., 2013b). In terms of kidney transplants, there are two main types of complement mediated attack on transplanted grafts; hyperacute rejection (HAR) or acute antibody-mediated rejection (AAMR), both of which occur due to activation of the classical complement pathway. One way of avoiding these rejection methods is to avoid HLA-mismatched grafts, which has been widely achieved in HAR by application of cross-matching and avoiding ABO-mismatched donors, but in AAMR this is still an issue. This is due to the large amount of patients presenting with HLA-pre-sensitisation due to blood transplants or previous transplants (Truong et al., 2007). One of the current treatment options for AAMR include the use of Bortezomib as a desensitization therapy to temporarily eliminate pre-existing donor specific antibodies (DSA) which bind to the endothelial cells of the graft. Bortezomib acts to stop the production of more DSA (Cicora et al., 2013), and is also widely used for treatment of multiple myeloma (Field-Smith et al., 2006).

1.5.1.2.2.2 Alzheimer's Disease

Alzheimer's disease (AD) is a neurodegenerative disease characterised by accumulation of β -amyloid plaques, formation of neurofibrillary tangles and loss of neurons (Fonseca et al., 2011). Inflammatory mechanisms have also been identified as operating within the brain in response to disease and injury, leading to AD, with C1q being identified as contributing to fibrillar plaques and tangles (Afagh et al., 1996). In AD, complement is triggered via C1q-mediated activation of the classical complement pathway, upon amyloid plaque accumulations (Webster et al., 2000), while a more recent study has identified C3-independent cleavage of C5 could also play a role (Fonseca et al., 2011). Due to this association between complement activation and AD, the use of steroidal and non-steroidal anti-inflammatory drugs, have been analysed. Drugs such as aspirin and ibuprofen have been tested in clinical trials with varied levels of success (Dokmeci, 2004, Jaturapatporn et al., 2012, Pasqualetti et al., 2009).

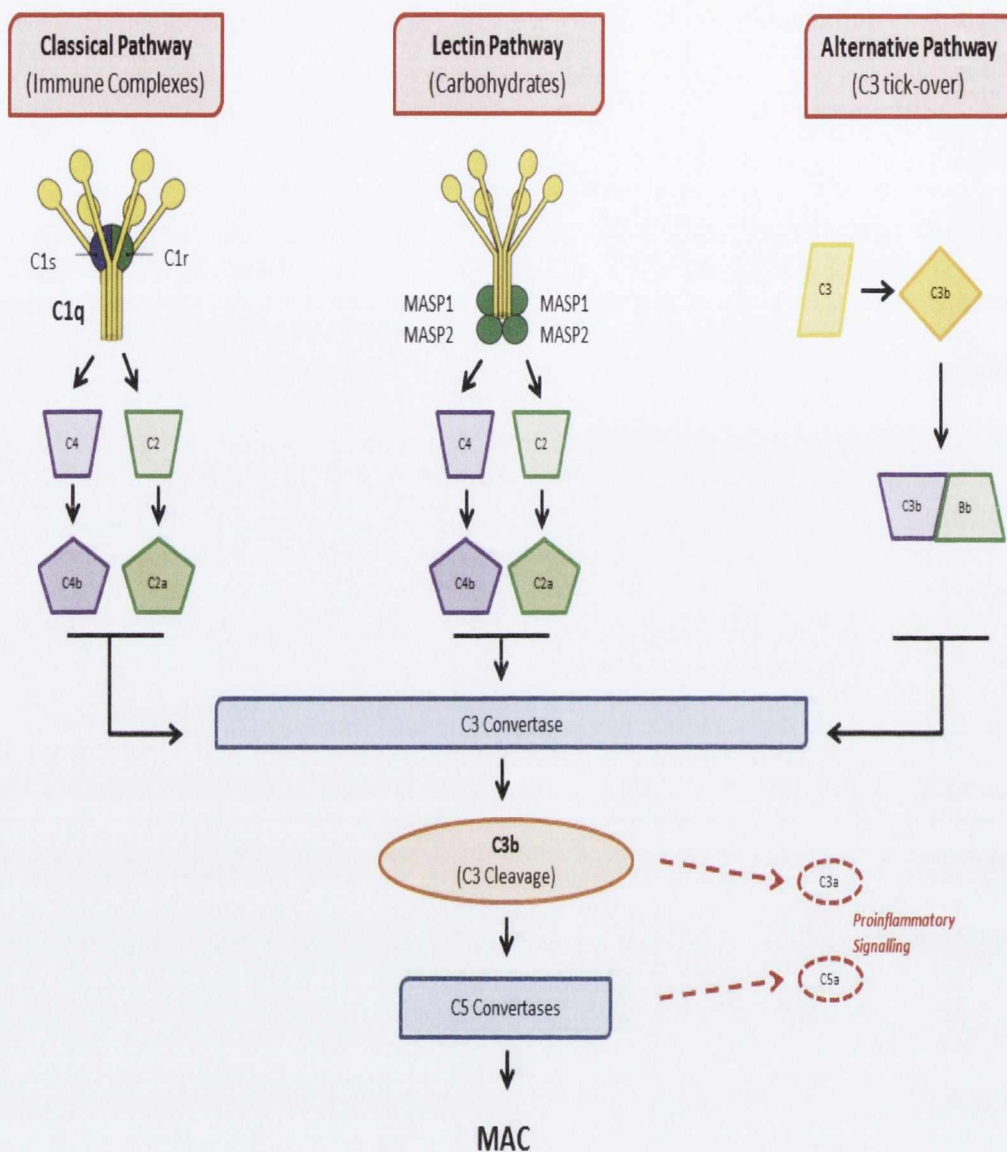


Figure 1.5: Complement activation with classical, alternative and lectin pathways. In the classical pathway C1 binds to the antigen-antibody complex; the lectin pathway is initiated by plasma lectin binding to microbes; the alternative pathway is activated by C3b binding to various surfaces including microbial cell walls. All three pathways result in C3 convertase, releasing C3b, which in turn combines with either C4b/C2a or C3b/Bb, which then form an enzyme to cleave C5. During C3 and C5 cleavage, C3a and C5a are released respectively. When these anaphylatoxins bind to their receptors (C3aR and C5aR, respectively) the immune reaction is triggered. (*Adapted from (Wood, 2004)*)

1.5.1.2.2.3 Systemic Lupus Erythematosus

Systemic lupus erythematosus (SLE) is the most well-known disease associated with complement activation. It is characterised by the deposition of immune complexes in tissues and it has been shown that hereditary deficiency of C1q results in the clinical manifestation of SLE. Deficiency of C4 or C2 can also induce SLE onset, but to a lesser extent than C1q (Pickering and Walport, 2000). These results suggest that the classical complement pathway may play a key physiological role in protection against SLE development. Detection of serum complement levels is commonly used as a marker for SLE, and during periods of disease activity the levels of serum complement are reduced. This is confirmed in patients suffering from severe SLE in which C1q, C1r and C1s levels are markedly low, which may also be accompanied by a reduced level of C3 (Pickering and Walport, 2000). Overall, the study of complement in SLE is complicated, and although it is known that it plays a role in SLE activation, the mechanisms behind which it exerts its effects remain unclear.

1.5.1.3 The Adaptive Immune Response

The adaptive immune response requires the clonal expansion of antigen-specific lymphocytes in response to external antigenic stimulation. It uses a process of somatic cell gene rearrangements to generate an army of antigen receptors that can identify fine distinctions between closely related molecules. Adaptive immunity can also remember the specific antigen, therefore making response to a repeat exposure more efficient. It is mediated by immunoglobulins and T cell receptors (TCRs), which are produced via recombination of variable (V), diversity (D) and joining (J) gene segments in vertebrates which depends on the recognition of recombination signal sequences (RSSs). This occurs in T- and B-cell progenitors respectively and is

mediated by recombination-activating gene 1 (RAG1) and RAG2 (Mogensen, 2009, Oettinger, 1992). Immunoglobulins are the antigen receptors on B-cells and there are five classes of immunoglobulin molecules recognised in humans; IgG, IgA, IgM, IgD and IgE. Immunoglobulins (Ig) can bind free intact antigens, while TCR only binds processed antigenic fragments that are presented on molecules encoded in the major histocompatibility complex (MHC) (Bartl et al., 2003). There are two classes of TCR; $\alpha\beta$ TCR and $\gamma\delta$ TCR, which are both heterodimers. The function of $\alpha\beta$ TCRs is reliant on major histocompatibility complex (MHC) class I and class II molecules which are expressed by antigen-presenting cells (APCs), however, $\gamma\delta$ TCRs can function independently of MHC class I and class II molecules. The role of T- and B-cells has been widely studied in Non-Hodgkin's Lymphoma and recent findings have shown that the use of an immunoglobulin-like CD20-Flex bispecific protein (BiFP), can induce tumour-specific T-cell immunity increasing survival rates in mice (Zhao et al., 2013) and that the presence of T cells could prevent the development of lymphoma in mice caused by reduction of Blimp1 or overexpression of Bcl6 in the B cell lineage (Afshar-Sterle et al., 2014).

1.5.1.4 The Role of the Major Histocompatibility Complex in the Immune Response

Tumour-associated macrophages (TAM) are the most abundant immune cell types found within the tumour microenvironment, and there are two main types; M1 and M2. M1 macrophages are activated by IFN- γ and express high levels of pro-inflammatory cytokines including IL-1 and IL-6 as well as MHC molecules, while M2 macrophages can be activated by IL-4 and IL-10, and can down regulate MHC class II molecules (Grivennikov et al., 2010). The 'major histocompatibility complex'

(MHC) region on chromosome 6p21 is involved in the immune response, contains genes that are involved in antigen recognition, and can mediate the adaptive immune response through $\alpha\beta$ TCRs as previously outlined. There are three classes of MHC genes; class I, class II and class III. MHC class I and II genes are human leukocyte antigen (HLA) genes which are mainly involved in adaptive immunity, and the MHC class III genes are contained within the same region, 6p21, between the class I and class II genes. These genes include genes coding for proteins involved in innate immunity such as complement factors C2, C4A and C4B, along with TNF- α and NOTCH4 and are initiated by the downstream TLR-mediated immune response (Xie et al., 2003). A recent study to investigate if MHC II molecules intersect with TLR's showed that mice deficient in MHC II molecules reduced the production of TLR-triggered pro-inflammatory cytokines in macrophages and dendritic cells (DC), suggesting that MHC II molecules play a non-classical role in the innate immune response as mediated by TLR's (Liu et al., 2011).

The first genome wide study in BO was undertaken in 2012, and identified an association between BO and the MHC region. This study identified variants at two different loci, 6p21 and 16q24, which were directly genotyped in both Irish and UK cohorts (Su et al., 2012). SNP analysis of these two loci were then taken from an international consortium, the Barrett's Esophagus and Esophageal Adenocarcinoma Consortium (BEACON) consisting of 2,398 cases and 2,167 controls from; European, Australian and US individuals that have confirmed European ancestry. Results indicated a significant association between these loci and BO, with $P = 4.09 \times 10^{-9}$ for the SNP at 6p21 (rs9257809) and $P = 2.74 \times 10^{-10}$ for the SNP at 16q24 (rs9936833). The SNP identified at 6p21 is located at the telomeric edge of the MHC region

between olfactory receptor genes OR2D12 and OR2D13, but the authors suggest that caution must be taken when investigating these underlying genes, especially in relation to the variant at 6p21, as results have shown that the linkage disequilibrium is long-range suggesting that a countervailing force may be present. However, this study has generated direct evidence suggesting that BO etiology has a genetic component with association with a region of the genome strongly associated with immune responses.

1.5.2 Cancer-Related Inflammation

There are now seven hallmarks of cancer identified (Figure 1.2), with the inflammatory microenvironment being recently included. The creation of a tumour-promoting microenvironment is an important step in initiation and growth of tumours, while injury to cells following exposure to an irritant also promotes tumour progression (Figure 1.6).

Genomic instability is defined as a process prone to genomic changes or an increased propensity for genomic alterations that occurs during the life cycle of cells (Shen, 2011). Cell division for most non-cancerous somatic cells aims to accurately duplicate the genome and evenly divide the duplicated genome into two daughter cells, ensuring the daughter cells will have exactly the same genetic material as the parent cell. In normal tissues, cell divisions are tightly regulated to help avoid neoplastic transformation, but if an abnormally high-frequency of errors occurs during cell division which are not repaired, then various forms of genome alterations can be noted in the daughter cells leading to tumourigenesis. The alteration of essential genes in a progenitor cell can transform a normal cell into a pre-cancerous cell, and although

the pre-cancerous cells may not be clinically considered as cancer, additional genomic changes would allow some of them to gain further growth advantages. Thus accumulated mutations in epithelial cells can lead to dysregulation of their growth and migration and they may also signal to recruit leukocytes (Lin and Pollard, 2004). Chronic inflammation can lead to tissue damage, genomic instability and increased mutation rates, and if these changes accumulate in excess, then the chronic inflammatory environment can initiate carcinogenesis (Coussens and Werb, 2002, Kidane et al., 2014). Thus the balance between both the pro- and anti-inflammatory immune responses during inflammation is important in determining the fate of the cell. Repair pathways have evolved to process DNA damage, but they are not perfect, which can result in the induction of mutations and genomic instability. For example, exposing mouse embryonic stem cells to nitric oxide, which is produced by activated macrophages and toxic to neighbouring cells, can induce homologous recombination between direct repeat sequences (Kiziltepe et al., 2005). Genomic instability can also be promoted by *H. pylori* by increasing the number of mutations in both the nuclear and mitochondrial DNA of mammalian cells, which has been implicated in the increased risk of developing gastric cancer (Carew and Huang, 2002, Machado et al., 2010).

Tumour cells can also produce cytokines and chemokines to attract immune cells to facilitate cancer development. Cytokine signalling can contribute to the progression of tumours in two possible ways; by stimulation of cell growth and differentiation and/or the inhibition of apoptosis of altered cells at the inflammatory site (Pollard, 2004, Hudson et al., 1999). Chronic inflammation is caused by prolonged exposure to a stimulus, therefore activating the immune response over a prolonged period of time

with the release of pro-inflammatory cytokines such as IL-6 and IL-1 β which accumulate *in situ* during chronic inflammation (Lin and Karin, 2007, Coussens and Werb, 2002). Cytokines encourage inflammatory activity by increasing the activity of leukocyte adhesion molecules on endothelial cells close to the affected site thereby attracting more immune cells and they may also function as pluripotent inducers of cellular proliferation (Silver and Hunter, 2010, Lin and Karin, 2007). Therefore, tumour initiation can be enhanced by production of cytokines and growth factors.

Production of cytokines such as TNF and IL-1 β can also be initiated by PRR's e.g. TLRs, resulting in a pro-inflammatory response (Newton and Dixit, 2012) with secretion of TNF α for example which can result in NF- κ B activation, a known pro-inflammatory transcription factor. As previously mentioned, *Helicobacter pylori* infection can be a contributing factor of gastric cancer (Cavaleiro-Pinto et al., 2011) and development of noncardia cancer, which can occur in all areas of the stomach except the gastroesophageal junction, in particular is associated with *H. pylori* infection where it can induce neoplastic changes in the epithelium (Peek and Blaser, 2002). Almost half of the population are affected by *H. pylori*, but only 1-3% progress to gastric cancer; thus it is been postulated that disease progression of *H. pylori*-infected individuals is dependent on both the presence of bacterial virulence factors and also the types of host responses. In relation to gastric mucosa-associated lymphoid tissue (MALT) lymphoma, a role for cytokines has been highlighted. Gastric tumour tissue obtained from the lymphoma contains higher amounts of Gastrin, a peptide hormone which stimulates secretion of gastric acid, than gastric antral mucosa (Konturek et al., 2000). In turn, this same study showed a significant increase ($P < 0.05$) in plasma levels of tumour necrosis factor (TNF) and interleukin 8

(IL8), in MALT lymphoma patients as compared to controls. A more recent study suggested that gastric MALT lymphomas contain high levels of APRIL-expressing macrophages (Munari et al., 2011). APRIL is a proliferation inducing ligand which is a member of the TNF family and is expressed by a variety of immune cells. Thus, *H. pylori* can trigger the release of APRIL by human monocyte-derived macrophages and T cells specific to *H. pylori* can contribute to the APRIL-enriched environment in MALT lymphoma.

1.5.2.1 Genetic Contributions to the Inflammation-Cancer Sequence

As highlighted in Figure 1.6, reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI) can induce DNA damage and genomic instability. Chronic inflammation can induce oxidative stress and damage, as well as lipid peroxidation (LPO), resulting in the production of excess ROS and RNIs within the affected cell (Bartsch and Nair, 2006). Many transcription factors, including STAT3 and HIF1 α can also be activated by oxidative stress or an inflammatory stimulus, as demonstrated in human lymphocytes, in which STAT3 activation and nuclear translocation was induced by oxidative stress following treatment with hydrogen peroxide (Carballo et al., 1999). Mutations within p53 can also be caused by oxidative damage as highlighted in colitis-associated cancer (CAC) suggesting that inflammation can cause genomic changes, for example research has highlighted that a reduction in p53 expression correlated with oxidative-stress induced apoptosis in a murine model of CAC (Werner et al., 2014), while in head and neck squamous cell carcinoma, loss of p53 can increase ROS, thus driving carcinogenesis (Skinner et al., 2012). Another study in transgenic mice expressing Hepatitis B surface antigen using adoptively transferred immune cells has also highlighted the role of inflammation (Guidotti et al.,

1995), indicating the importance of inflammation in the pathogenesis of liver tumours induced by HBV and other hepatic irritants (Chisari et al., 2010). Both of these examples highlight the role of inflammation in cancer, by promoting proliferation and invasion, while decreasing apoptosis.

Cytokines can activate transcription factors including STAT3 and NF κ B which in turn induce genes that stimulate cell proliferation and survival, angiogenesis, invasion, motility, and chemokine and cytokine production. Both of these transcription factors have been identified as nonclassical oncogenes as their activation in tumourigenic cells does not generally result in direct mutations. The Wnt signalling pathway can also contribute to cancer by regulating cell growth and survival through activation of β -catenin. All of these pathways are widely reported to be involved in the inflammation-cancer sequence of various cancers and will be discussed in more detail throughout the following sections.

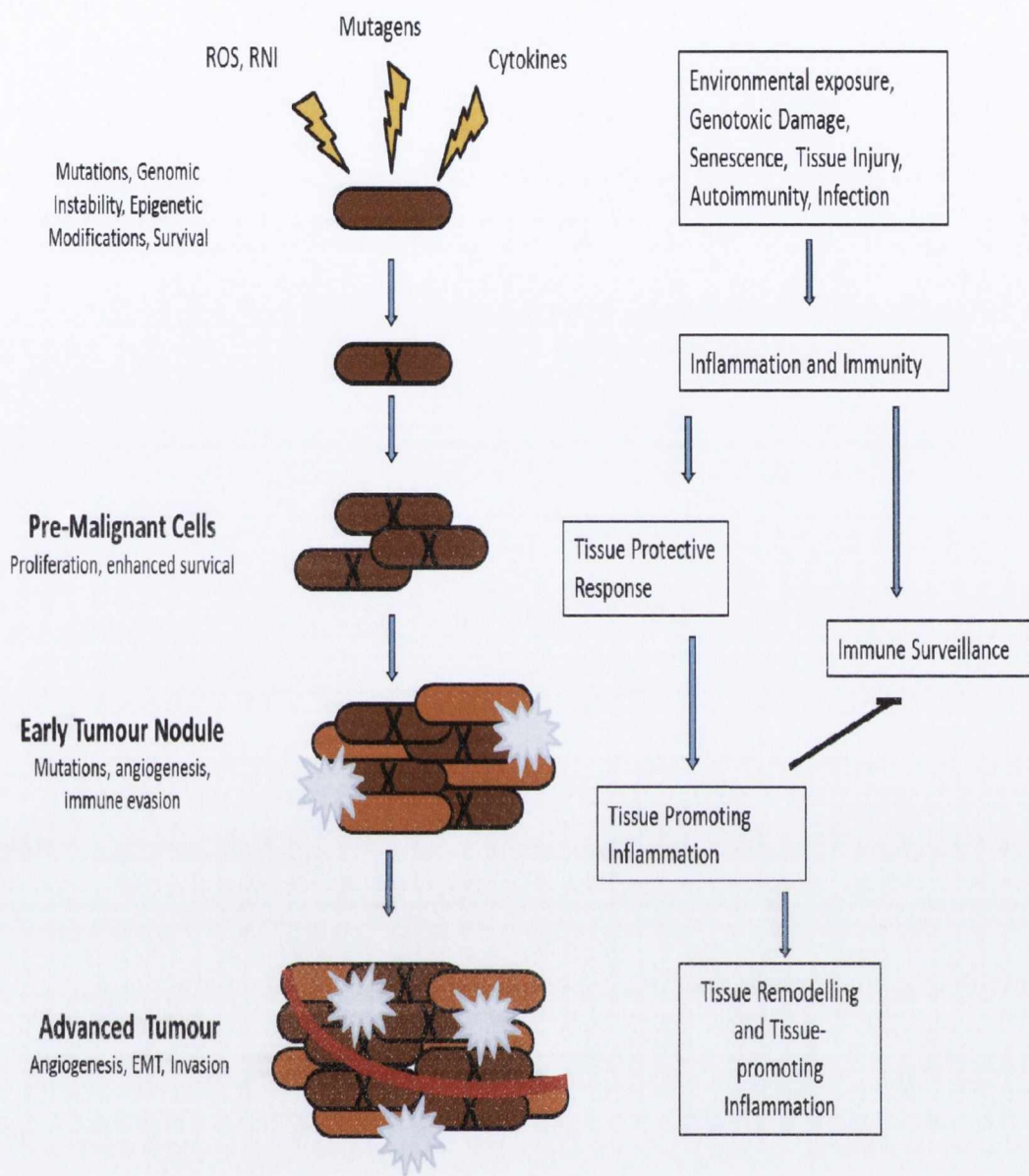


Figure 1.6: The Role of Inflammation in Cancer. Inflammation can contribute to tumour initiation via mutations, genomic instability and epigenetic modifications to promote cell survival. Inflammation can induce cell proliferation and survival, leading to mutations, angiogenesis and immune evasion. Mutated cells are marked with an "X". Orange cells indicate stromal cells. Immune and inflammatory cells are indicated in blue, and the red curve indicates a blood vessel. ROS: reactive oxygen species; RNI: reactive nitrogen intermediates; EMT: epithelial-mesenchymal transition. (Modified from (Grivennikov et al., 2010))

1.5.2.1.1 Nuclear Factor kappa-light-chain-enhancer of Activated B cells (NFκB) Pathway

In 1986 the transcription factor, NFκB, was identified as a nuclear factor that can bind to the enhancer element of the immunoglobulin kappa light-chain of activated B-cells (Sen and Baltimore, 1986). There are five members of the NFκB family, p65 (RelA), RelB, c-Rel, p105/p50 and p100/p52 (Ghosh and Karin, 2002), and in most cells, the NFκB proteins can exist as hetero- and homodimers in the cytoplasm which are bound to inhibitory proteins called IκBs (inhibitor of κB). Upon cellular stimuli, NFκB can be activated through either of two pathways; canonical or non-canonical. The canonical NFκB pathway is of key importance in the initiation and progression of tumours and within this the pathway NFκB activation is dependent on the IκB kinase complex (IKK), which has two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, IKKγ or NEMO. When stimulated, IκBα is phosphorylated at Ser32/36 by IKK resulting in degradation of IκBα and the release of the p65-p50 dimer where it accumulates in the nucleus. The non-canonical NFκB pathway is regulated by an IKKα homodimer and in this cascade, RelB-p100 heterodimers can be processed to RelB-p52 heterodimers. Within the nucleus, NF-κB dimers can activate target genes such as those involved in cell cycle regulation (cyclin D1), suppression of apoptosis (Bcl-2 and Bcl-xL) and inflammation (IL-6 and IL-8). NFκB is also known to play a central role in cancer initiation and progression, while its role in immune defence is to target and eliminate transformed cells, correlating with high activity of cytotoxic immune cells against cancer cells. For example, NFκB is essential for increased T-cell recruitment for tumour rejection in murine lung tumours (Hopewell et al., 2013) and it is constitutively activated in many cancer types, including prostate and lymphoma, thereby exerting a pro-tumourigenic function (Palayoor et al., 1999, Davis et al.,

2001). This results in up-regulation of anti-apoptotic genes which can induce a cell proliferation and survival mechanism. Activation of NF κ B can induce pro-inflammatory cytokines including IL-1, IL-6 and IL-8, which in turn elevate NF κ B activity, leading to a positive feedback loop (Ben-Neriah and Karin, 2011).

NF κ B can play also an oncogenic role in intestinal epithelial cells which is suggested to be mediated via its anti-apoptotic function, possibly through induction of Bcl-xL, which can prevent the apoptotic elimination of premalignant cells (Greten et al., 2004). For example, in a murine study, it has also been shown that IL-6, a multifunctional NF κ B regulated cytokine, is produced by lamina propria macrophages and dendritic cells in colitis-associated cancer. Inhibition of IL-6 reduces the quantity and size of colonic adenomas in mice treated with a combination of azoxymethane (AOM) and dextran sulphate sodium (DSS), which was shown using wild-type controls, and IL-6^{-/-} mice (Grivennikov et al., 2009). These studies highlight the role of NF κ B in the production of cytokines that have a known association with cancer including IL-6.

1.5.2.1.2 Signal transducer and activator of transcription 3 (STAT3)

Pathway

There are seven 'signal transducer of transcription' (STAT) proteins, which act as transcription factors to facilitate signalling between cytokines and growth factor receptors. They are activated by phosphorylation resulting in stabilisation of homo- and/or heterodimers in the cytoplasm of the cell, which then translocates to the nucleus (Shah et al., 1998). Once here, the phosphorylated STAT binds to consensus

DNA elements resulting in activation and transcription of specific genes (Dvorakova et al., 2004).

Each of the STAT proteins has an affinity to a particular cytokine family; for STAT3 this is the IL-6 family, which is associated with cancer-promoting inflammation (Jarnicki et al., 2010). The IL-6 family of cytokines is defined by the shared use of the gp130 receptor by IL-6, LIF, OSM, CNTF, CT-1, IL-27 and IL-11 cytokines. When gp130 is triggered, activation of Jak1, Jak2 or Tyk2 results in subsequent tyrosine phosphorylation of gp130. As with NF- κ B, STAT3 activation can result in a number of cellular outcomes; survival, proliferation, angiogenesis, metastasis, cell adhesion, inflammation and cell signalling, each of which targets different genes. For example, apoptosis is inhibited by STAT3-mediated up-regulation of Bcl-2 proteins, which can be reversed by treatment with an ALK inhibitor drug, Crizotinib (Hamedani et al., 2014). STAT3 is also known to be aberrantly activated in several cancers which originate within epithelial cells, including breast and colon cancers (Zhang et al., 2013a, Wang et al., 2013a).

Activation of the transcription factor, STAT3, is generally dependent on a multitude of growth factors and cytokines including IL-6 and IL-11 within the cancer setting (Bollrath et al., 2009). It induces expression of cytokines, and growth and angiogenic factors, which in turn activate the STAT3, thus establishing a feedback loop between both tumour and immune cells within the tumour microenvironment which can result in the persistent activation of STAT3. The tumour-promoting effect of the IL-6 cytokine is generally exerted by the STAT3 transcription factor in many tumour types including breast and colitis-associated cancers (Becker et al., 2004, Berishaj et al.,

2007, Bollrath et al., 2009) including a mouse model of CAC, in which STAT3 was inhibited in enterocytes, resulting in reduced tumour growth (Bollrath et al., 2009).

1.5.2.1.3 Wnt Signalling Pathway

Wnt proteins are secreted ligand proteins of which 19 have been identified in humans. The canonical Wnt pathway results in stabilisation of β -catenin resulting in translocation from the plasma membrane to the cytoplasm and mainly accumulates in the nucleus. Secreted Wnt proteins can bind to either the Frizzled family of receptors or the single pass transmembrane low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) both of which transduce a signal to a number of intracellular proteins including Dvl, GSK3, APC and Axin. When bound, these proteins form a β -catenin degradation complex which facilitates the recognition of phosphorylated β -catenin by β -TrCP, leading to its ubiquitination and subsequent degradation (Liu et al., 1999). Activation of the Wnt pathway results in recruitment of Axin to LRP5/6 and inhibition of GSK-3 β by Dvl, resulting in β -catenin phosphorylation (Cliffe et al., 2003), while subsequent stabilisation of the cytoplasmic β -catenin results in the translocation to the nucleus, where it acts as a transcription factor (Olmeda et al., 2003). This results in the formation of the β -catenin/TCF transcription complex, where the β -catenin binds to the TCF/LEF family members, leading to activation of the target genes such as MMP-7, c-Myc and PPAR- δ , which are responsible for both cell proliferation and differentiation (Nelson and Nusse, 2004). It has also been identified that, when Wnt signalling does not occur, APC (adenomatous polyposis coli) can down-regulate β -catenin, by preventing the formation of the β -catenin complex (Gatalica et al., 2013). Thus, the use of siRNA to inhibit APC expression has

been shown to increase the levels of cytoplasmic β -catenin, resulting in increased translocation of β -catenin to the nucleus (Reed et al., 2008).

Uncontrolled β -catenin activation acts to promote cell survival and induce DNA damage, oncogene-induced senescence (OIS), and apoptosis of immature thymocytes. (Xu et al., 2008). The role of Wnt/ β -catenin in the inflammation-cancer sequence has been extensively reported and has been widely studied in colon cancer. Recently Wnt/ β -catenin signalling in effector T-cells and/or T-regulatory cells was shown to promote inflammation and initiate colon cancer (Keerthivasan et al., 2014), while another study indicates that TNF- α can induce the expression of APC, AXIN2 and GSK3 β (Wnt components) by downregulating CDX2 expression, which may have a tumour-promoting effect on colon cancer cells (Coskun et al., 2014). Another study indicated the role of β -catenin in HCC, in which Wnt signalling was shown to induce an inflammatory response in hepatocytes involving both direct transcriptional control by β -catenin and activation of the NF- κ B pathway (Anson et al., 2012).

1.5.2.2 The Role of Inflammation in Oesophageal Cancer

Several genes play key roles in immunity, and the development of inflammation progressing to cancer. Activation of NF κ B, a marker of inflammation, and STAT3 pathways play central roles within oesophageal carcinogenesis (Dvorak et al., 2007, O'Riordan et al., 2005) and trigger activation of cytokines including IL-6 and IL-8 (Picardo et al., 2012). Rather than being activated by a direct mutation, both NF- κ B and STAT3 are activated by signals produced by surrounding cells, such as the previously mentioned feedback loop between IL-6 and STAT3. Both NF- κ B and STAT3 can interfere with p53 synthesis, whose inactivation is known to play a key

role in chromosomal instability, inflammation and cancer (Tomasini et al., 2008, Colotta et al., 2009). In a transformed BO cell line (BAR-T), activation of the NF- κ B pathway can induce resistance to apoptosis (Hormi-Carver et al., 2009), and phosphorylated STAT3 is also present in this cell line, indicating an increase in STAT3 signalling compared to normal oesophageal cells. STAT3 activation has also been shown in clinical samples of both BO and OAC (Zhang et al., 2011), while it can also play a key role in oesophageal carcinogenesis, via regulation of Oct-1 (Wang et al., 2013c).

1.5.2.2.1 NF κ B in Oesophageal Cancer

Clinical studies have shown that NF- κ B is highly expressed in both BO and OAC, as are the pro-inflammatory cytokines IL-8 and IL-1 β (O'Riordan et al., 2005), and the bile acid deoxycholic acid (DCA) can also induce IL-8 expression and activate the NF- κ B pathway in OAC cell lines *in vitro* (Jenkins et al., 2004). NF- κ B is also expressed in OSCC in conjunction with XIAP, a protein belonging to a family of apoptotic suppressors, both of which have been identified in clinical samples (Zhou et al., 2013). Cross-talk has been identified between NF- κ B and STAT3 in head and neck squamous cell carcinoma (HNSCC) cells indicating that IL-6 is down-regulated upon inhibition of NF- κ B. Furthermore it was shown that IL-6 expression, due to increased IL-6 promoter transcription, is dependent on intact NF- κ B being present upstream of the IL-6 transcriptional regulation site (Squarize et al., 2006). These studies demonstrate the role that NF- κ B plays not only in cancer but in oesophageal cancer, both OAC and OSCC, highlighting its importance in both the development and progression of the disease.

1.5.2.2.2 STAT3 Signalling in Oesophageal Cancer

Activated STAT3 is highly expressed in IHC samples of the OAC carcinogenic sequence and the levels of pSTAT-3 (Y705) increase from normal to OAC samples, with the highest levels recorded in high-grade dysplastic and OAC tissue samples (Dvorak et al., 2007), which is thought to be due to bile acid mediated inflammation (Figure 1.7). STAT3 activation was also noted in the OAC cell line, Seg-1, after treatment with pH 4 bile acids, as compared to either bile acids or pH 4 separately (Dvorak et al., 2007) demonstrating that, after the treatment of Seg-1 BO cells with bile acids at pH 4, the levels of IL-6 mRNA were statistically increased, and secreted IL-6 was found to be doubled (Dvorak et al., 2007). Thus IL-6 was shown to mediate DCA induced apoptosis in Seg-1 cells, therefore sensitising them to apoptosis (Dvorak et al., 2007).

IL-6 is a pleiotropic Th2 cytokine which activates STAT3 upon binding to its receptor, IL-6R, leading to over-expression of anti-apoptotic and angiogenic genes including Mcl-1 and Bcl-xL (Zhang et al., 2011). Over-expression of IL-6 is implicated in the pathogenesis of different tumour types including breast cancer, head and neck cancer, and colitis-associated cancer (Chang et al., 2013, Grivennikov et al., 2009, Yadav et al., 2011), indicating that inhibition of IL-6 can reduce tumour burden while increasing IL-6 levels accelerates tumour formation. It can also act as both a pro-inflammatory and an anti-inflammatory cytokine, playing a key role in the transition from acute to chronic inflammation (Hurst et al., 2001). Interestingly, colitis-associated cancer and OAC are both associated with chronic inflammation and IL-6 has been shown to be a highly secreted cytokine in BO biopsy samples as compared to oesophageal squamous epithelium (Dvorakova et al., 2004)

(Lukaszewicz-Zajac et al., 2012). On comparison of serum IL-6 levels to carcinoembryonic antigen (CEA) levels in OAC, it was found that IL-6 levels in OAC patient samples were nearly three times higher than CEA levels (Lukaszewicz-Zajac et al., 2012). Carcinoembryonic antigen is a glycoprotein involved in cell adhesion and is used as a marker for colorectal and oesophageal cancers (Bhatnagar et al., 2002). These studies highlight the major roles that the STAT3 pathway and the feedback loop with IL-6, play in oesophageal cancer and inflammation.

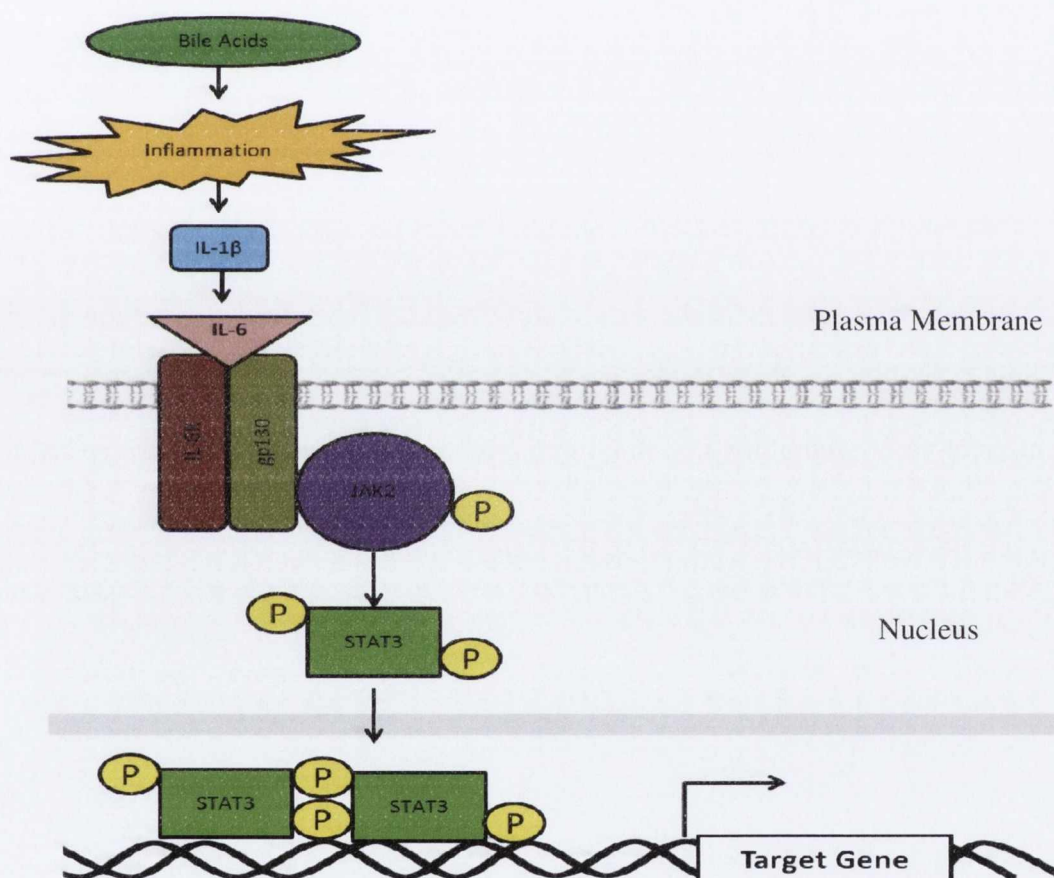


Figure 1.7: Bile Acid Mediated STAT3 Activation Occurring Within the Oesophageal Setting. Repeated acid reflux of bile acids can cause chronic inflammation of the cells lining the oesophagus. This triggers the release of proinflammatory IL-1 β which induces IL-6 production. IL-6 binds to its receptor IL-6R triggering gp130 homodimerisation. This activates JAK2 with subsequent tyrosine phosphorylation of STAT3. STAT3 activation then results in transcription of the target gene. (Adapted from (Li et al., 2013a).

1.5.2.2.3 The Role of Wnt/ β -catenin in Oesophageal Cancer

As previously outlined, Wnt/ β -catenin signalling plays a key role in colon cancer. The translocation of β -catenin to the nucleus can activate tumour growth promoting genes, which usually occurs early in the sequence from BO to OAC and is generally associated with the progression from metaplasia to LGD (Clement et al., 2006a). Although studies are conflicting about the level of nuclear accumulation of β -catenin in OAC (Bian et al., 2000, Wijnhoven et al., 2000, Osterheld et al., 2002), a more recent study from 2012 has shown that Wnt activation does promote dysplasia in BO (Moyes et al., 2012). This study demonstrated that in oesophageal squamous epithelium, an overlap between β -catenin and Ki67, a known marker for cell proliferation, could activate Wnt signalling, thus contributing to cell proliferation. The most interesting finding from this study was that high-grade dysplasia (HGD) showed a more marked increase in cytoplasmic and nuclear β -catenin than any other stage of the OAC sequence, including OAC itself (Moyes et al., 2012). This correlated with an earlier study showing that in BO tissue, a large accumulation of β -catenin was found in the nucleus of BO cells (Bobryshev et al., 2010). Mutations that stimulate constitutive activation of the Wnt pathway can induce cancer onset, for example an APC mutation in colorectal cancer (CRC), which increases the concentration of free β -catenin (Morin et al., 1997). While in BO and OAC, loss of heterozygosity of APC gene has also been identified in dysplastic BO (Jankowski et al., 2000).

1.5.3 Current Treatment Options

The use of chemotherapy with or without radiation therapy before surgery can, in theory, improve baseline dysphagia, help downstage a tumour and treat micro-metastatic disease that would not usually be detectable in imaging studies, while also

helping to indicate the response of a tumour to a specific treatment. The use of multi-modal treatment to increase long-term survival rates has been identified as an alternative option to individual treatments. Randomised trials have shown varied results in relation to the roles of chemotherapy and radiation therapy when used to improve surgical results.

In a study by the Medical Research Council Oesophageal Cancer Working group (2002), a cohort of 802 patients were randomised and treated with two cycles of cisplatin/5-FU followed by surgery vs surgery alone. Median (16.8 mo vs 13.3 mo; difference 107 d; 95%CI: 30-196) and 2-year survivals (43% vs 34%-difference 9%; 95%CI: 3-14, respectively) were clearly improved in the chemotherapy group, while the curative resection rate was also marginally improved from 55% to 60%. A meta-analysis performed of 12 randomised trials where pre-operative chemotherapy was used, the 5-year overall survival benefit was 4% (Gebski et al., 2007), while the benefit was smaller for OSCC as compared to OAC (4% vs 7%). There is only one meta-analysis for patients having pre-operative radiotherapy alone (Arnott et al., 2005), which involved 5 randomised trials and compared surgery alone with pre-operative radiotherapy followed by surgery. OSCC was the dominant histology type noted although some trials did include OAC. The findings identified improved survival in patients that received the combined therapy but was not statistically significant ($p=0.06$).

In the study by (Gebski et al., 2007), 10 randomised trials investigated tri-modality vs surgery alone, and 8 trials investigated pre-operative chemotherapy vs surgery alone. Tri-modal therapy indicated a 13% benefit in 2-year survival rates in both OSCC and

OAC, which suggests a synergistic benefit using neo-adjuvant chemotherapy plus radiotherapy for the treatment of oesophageal cancer. Another study supporting the use of multimodal treatment instead of unimodal, showed that patients treated with neo-adjuvant chemo-radiotherapy and surgery had an increased 5-year loco-regional control, (6.1% vs 22.1%), disease-free survival (40.7% vs 9.9%) and 5-year overall survival (52.6% vs 6.5%) rates, with a median survival of 62 months vs 12 months, when compared to patients treated with just chemo-radiotherapy alone (Liao et al., 2004). This overall finding, that multimodal treatment results in the best survival rates has also been identified in more recent studies, where the 5-year survival rates of T4 OSCC patients was 42% in patients treated neo-adjuvantly followed by surgery, as compared to 19% in patients who underwent surgery alone (Morita et al., 2012).

As outlined above, neo-adjuvant therapy can be extremely useful and is widely used for advanced oesophageal cancer. However, a large proportion of patients (60%-70%) does not respond well to neo-adjuvant therapy and can develop severe adverse effects (GebSKI et al., 2007). Given this, predictive markers for personal multimodal treatments could be of great benefit in oesophageal cancer treatment. Molecular biomarkers that can predict response to neo-adjuvant therapy have been investigated in multimodal treatments within an oesophageal setting and successful examples of biomarker have been identified (Uemura and Kondo, 2014). There are many different methods utilised to identify predictive biomarkers including microarray-based gene expression profiling which has identified genes including EPB41L3, RNPC1, RTKN, STAT5B and NMES1, which were subsequently validated via qRT-PCR (Maher et al., 2009). Other methods include utilising a panel of single nucleotide polymorphisms (SNP), with a number of studies identifying that

SNPs in ERCC1 holds great benefit as a predictive biomarker (Houtsmuller et al., 1999, Schneider et al., 2005, Kim et al., 2008, Warnecke-Eberz et al., 2009). Schneider et al show that upon neo-adjuvant treatment with 5-FU/cisplatin/36 Gy followed by surgery, the gene expression level of ERCC1 was significantly reduced ($p<0.01$), along with others including TS ($p<0.01$), DPD ($p=0.03$), GST-Pi ($p=0.03$), EGFR ($p=0.04$) and HER2 ($p<0.01$). Kim et al, then showed that ERCC1-negative patients (following multimodal therapy and surgery), had a tendency towards prolonged survival ($p=0.1$) and event free survival ($p=0.08$), although neither were considered significant. ERCC1-negative patients were shown to have an increased pathological response ($p<0.001$) as compared to ERCC1-positive patients. The study by Warnecke-Eberz identified that the SNP C118T in ERCC1 indicated a significant increase in therapy response ($p<0.003$), with the rarely occurring AA genotype indicative of a response in comparison to the GG or AG genotypes which were indicative of non-predictive and non-response respectively. Other methods include microRNA (miRNA), in which miR-296 was shown to be differentially regulated and showed a two-fold difference, while its inhibition also increased the chemo-sensitivity of oesophageal cancer cells to chemotherapeutic agents including 5-fluorouracil and cisplatin (Hong et al., 2010). There have been recent improvements in both the detection and treatment of oesophageal OAC, but despite this the overall survival of patients remains lower than many other solid tumours, thus further advances in treatment options is needed to improve both survival rates and quality of life of OAC patients.

1.5.3.1 Targeted Therapy for Cancer Treatment

Due to the number of patients that do not respond positively to chemotherapeutic therapies, and/or suffer from adverse side-effects, the development of specific, personal therapies are needed. Understanding the molecular basis of tumour initiation and progression is a basic starting point for the development of these targeted therapies, an example of which is, Herceptin (trastuzumab), and is already in use. The Her-2/neu oncogene, identified by Weinberg and Ullrich, has become a common target for the treatment of a specific type of breast cancer, in which the Her-2/neu is overexpressed. Ullrich and Slamon produced a monoclonal antibody, Herceptin, to treat tumours that overexpress the Her-2/neu gene, and acted to reduce the rate of cell proliferation while leaving normal cells unaffected. Overexpression of Her-2/neu has not only been identified in breast cancer, but several others including oesophageal squamous cell carcinoma (Wu et al., 2012), small-cell lung cancer (Minami et al., 2013), gallbladder (Toledo et al., 2012) and gastric cancer (Huang et al., 2013). Due to the success of this targeted therapy, the same idea is being applied to all other cancers in the hope of improving the quality of life of patients and increasing overall survival rates.

Biomarkers are also commonly investigated and utilised for the treatment of cancers including breast and prostate where they are commonly used in clinical determination of cancer progression. They have the potential to greatly improve the clinical management of patients with BO and OAC but have generally not entered mainstream clinical practice (Ong et al., 2010). Biomarkers are characteristics that can be objectively measured as indicators of normal biological processes, pathological processes or a response to a therapeutic treatment (Ong et al., 2010, Manne et al.,

2005). Biomarkers assayed in biological samples (blood or endoscopic biopsies) may be identified to improve clinical management at each stage in the disease, while screening biomarkers could also help identify cases of BO in the general population that may otherwise remain undiagnosed. Predictive biomarkers could also be used in combination with or even to replace current surveillance programs for detecting dysplasia, while also helping to predict patients that may be at a higher risk of developing cancer in the future. Additionally, biomarkers may also play a role in helping to determine patient treatment response. Examples of biomarkers that have shown success identifying BO patients that are at high risk of developing OAC are shown in Table 1.1. These biomarkers include the presence of high grade dysplasia (HGD), abnormalities in DNA content, loss of heterozygosity (LOH), methylation status, and proliferative or cell cycle markers.

For biomarkers to serve as risk factors or markers for early detection they must obey three specific criteria: firstly, the biomarker must be differentially expressed between normal, high-risk, and tumour tissues; secondly the marker and its assay must provide predictive accuracy for either risk or presence of cancer; and finally variance between detection tests and between laboratories must be known (Srivastava et al., 2001). Although a significant amount of biomarkers have been identified in cancer, most do not progress to clinical practice due to the fact that they do not obey by these criteria. In terms of the limitations associated with biomarker use, it has become apparent that a single biomarker is unlikely to predict disease progression accurately, due to the complexity of disease progression, thus the use of a biomarker panel may be the most accurate predictor of progression (Illig et al., 2013). Another limitation is the fact that the common use of these panels would be very expensive, and their routine clinical

use would not be feasible, in terms of cost, as well as a lack of technical expertise (Illig et al., 2013, Ong et al., 2010). Thus the practicalities of biomarker use should be a key consideration before further research and resources are put into this area.

Table 1.1: Most Promising Biomarkers for Identifying BO Patients at High Risk of Developing OAC

Biomarker	Risk of Progression to OAC	Limitations	References
HGD	Reid: RR 28 (95% CI: 13-63) Schnell: RR 12.1 (95% CI: 5-29.4)	- Progression risk ranges 16-59% - Many HGD patients show invasive OAC - Late in pathogenic process	(Reid et al., 2000, Schnell et al., 2001)
LOH & DNA Content	LOH 17p: RR 10.6 (95% CI:5.2-21.3) LOH 9p: RR 2.6 (95% CI: 1.1-6.0) Aneuploidy: RR 8.5 (95% CI: 4.3-17.0) Tetraploidy: RR 8.8 (95% CI: 4.3-17.7) Biomarker Panel: RR 38.7 (95% CI: 10.8-138.5)	- LOH detection is complex - Expensive	(Galipeau et al., 2007, Reid et al., 2000)
p53 Positive (IHC)	OR 11.7 (95% CI: 1.93-71.4)	P53 staining does not always correlate with mutations	(Murray et al., 2006)
Mcm2	OR 136 (95% CI: 7.5-2464)	This was small study, larger study needed	(Sirieix et al., 2003)
Cyclin A	OR 7.6 (95% CI: 1.6-37)	Larger study needed	(Lao-Sirieix et al., 2007)
Hypermethylation: p16 Runx3	OR 1.74 (95% CI: 1.33-2.2) OR 1.8 (95% CI: 1.08-2.81)	Methods are technically complicated & not cost effective	(Schulmann et al., 2005)

(* Table adapted from(Ong et al., 2010, Illig et al., 2013))

1.5.3.1.1 Gene Inhibition as a Method of Targeted Therapy

Gene inhibition can help elucidate the role of a gene(s) on a number of cellular functions including; proliferation, invasion, migration and apoptosis and can utilise RNA interference (RNAi) which will be discussed further in section 1.6. For example, to study of the role of Nanog inhibition on breast cancer cell proliferation, short hairpin RNA (shRNA) was utilised, resulting in significantly reduced cyclinD1 protein in two breast cancer cell lines; MCF-7 and MDA-MB-231 (Han et al., 2012). The use of RNAi can also be harnessed to analyse the effect of gene inhibition on drug efficacy. For example, small interfering RNA (siRNA) targeting HIF-1 α can increase the cytotoxicity of cisplatin in OSCC cells (Liao et al., 2012), and it has also been identified as a key mediator of cellular adaptation to hypoxia, by activating metabolic and signalling pathways which promote cell survival. Stabilisation of HIF-1 α protein can also be induced by the pro-inflammatory cytokines IL-1 β and TNF α which suggests that HIF-1 α may play a role in inflammation (Jung et al., 2003).

In relation to targeted therapy aimed at the inflammatory response, it is hypothesised that selective targeting/interference of genes known to be involved in inflammation may provide a therapeutic method for targeting of pro-apoptotic and anti-inflammatory cytokines. For example mutations have been identified in NOD2 locus, which can lead to Crohn's disease and colorectal cancer (CRC) resulting in increased production of IL-1 β , which is known to be pro-inflammatory (Lin and Karin, 2007).

1.6 RNA Interference

RNA interference was first noted in plants, fungi and worms, after the addition of a control sense oligonucleotide into a cell which decreased gene expression (Fire et al., 1998a, Mello and Conte, 2004, Grishok and Mello, 2002, Tabara et al., 1999). Craig Mello and Andrew Fire received the Nobel Prize in Physiology or Medicine in 2006 for their independent determination of specific gene interference by double stranded RNA (dsRNA) in *C. elegans* and *A. thaliana* (Fire et al., 1998a). In *C. elegans*, it was demonstrated that injection of dsRNA using sequence homology to target a specific gene, could result in inhibition of the expression of that gene with the expected phenotype having already been identified following a loss of function mutation. The results of the dsRNA experiment were deemed highly specific when compared to the loss of function mutation phenotype concluding that the mechanism by which genetic silencing was achieved, was post-transcriptional, therefore resulting in a reduction in mRNA levels of the target gene (Fire et al., 1998a). Subsequently, Mello identified that the mutant *rde-1* gene, which is resistant to RNAi and a member of the *piwi/sting/argonaute/zwille/eIF2C* gene family within *C. elegans* is conserved throughout phyla including *A. thaliana* (Tabara et al., 1999). In plants, the *rde-1* homolog is *argonaute 1 (ago-1)*, which binds to specific miRNAs to determine the fate of cells involved in the formation of both the top and bottom of the leaf (Bohmert et al., 1998). Loss of post-transcriptional gene silencing (PTGS) had previously been reported in plants by mutant *sgs* genes (Elmayan et al., 1998), but the work by Mello identified a conserved ancient genetic link from plants to vertebrates, indicating that RNAi, like PTGS, may not be necessary and may even be an acquired defence mechanism. The impact of these studies has led to the demonstration that a conserved mechanism exists in fungi, plants and animals, an understanding of which has enabled

us to unravel dsRNA and target mRNA degradation by the use of complementarity of the resultant single strand (Mello and Conte, 2004).

In mammalian cells, gene interference is initiated when long dsRNA is cleaved by the RNase III enzyme, Dicer, into short double stranded RNA (dsRNA), which are between 20-25 nucleotides long. There is usually a two base pair overhang at the 3' end which allows binding of Dicer, which contains two RNase domains and one PAZ domain, to the 5' phosphate of the dsRNA. Dicer catalyzes the first step within the RNAi pathway and initiates formation of the RNA-induced silencing complex (RISC). The catalytic component, *argonaute*, is an endonuclease which can degrade messenger RNA (mRNA), whose sequence is complementary to that of the siRNA guide strand. The double stranded siRNA is then unwound, and the guide strand is taken up by RISC complex. The single siRNA strand then acts to guide the endonuclease activity of the activated RISC complex to the complimentary site on the mRNA, resulting in the mRNA being cleaved, and degradation of the passenger strand (Figure 1.8) (Ambesajir et al., 2012).

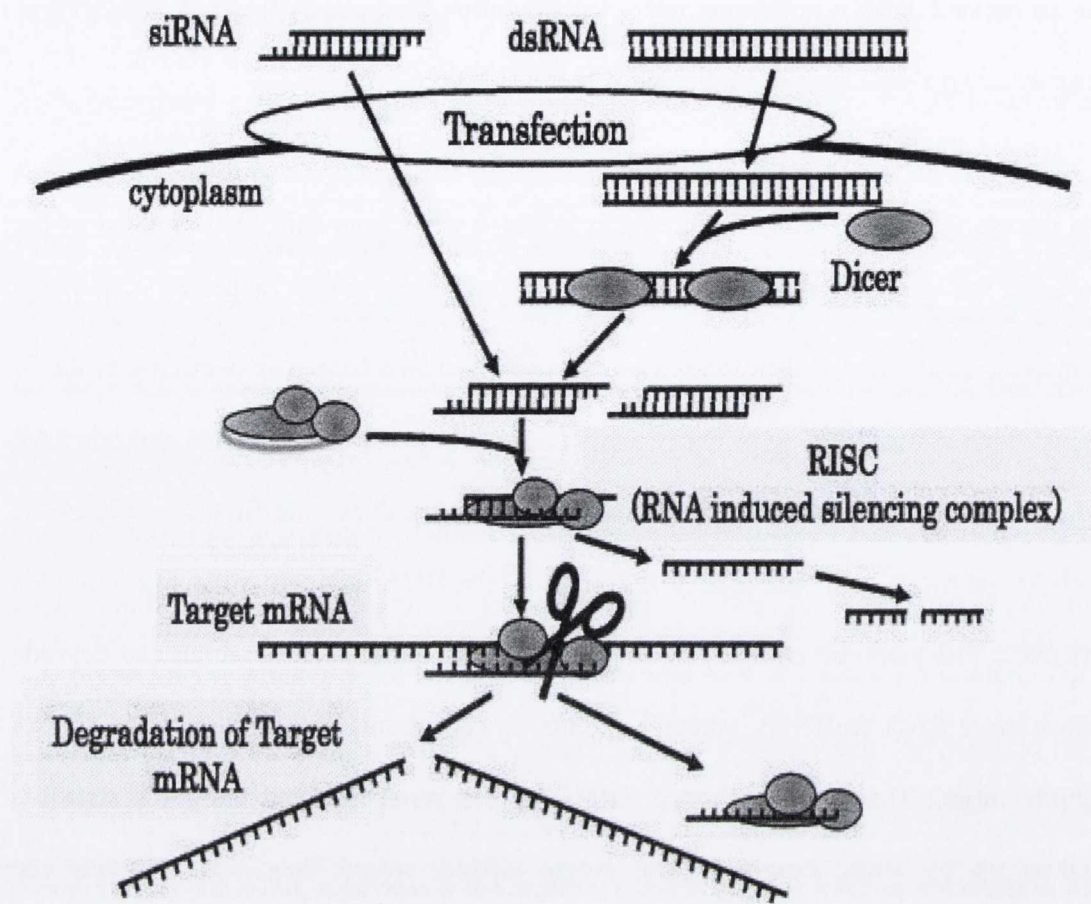


Figure 1.8: RNA Interference Pathway. External dsRNA or siRNA may be added to a cell via commercial reagent, for transfection into the cells using a lipid-based reagent, to exert its ability to knock down the intended gene of interest, through cleavage by Dicer (*Kawata et al., 2011*)

This method of unravelling dsRNA and utilising the individual strands to target mRNA degradation was identified in humans using small interfering RNA (siRNA), which are double-stranded RNA molecules of between 21-22 nucleotides in length. They are synthesised from double-stranded segments of matched mRNA through RNA-dependent RNA polymerase, resulting in specific gene interference by degradation of mRNA. MicroRNA (miRNA) on the other-hand, are single stranded, small non-coding RNA molecules of about 19-25 nucleotides in length, that can be found in plants, animals, and some viruses, and are involved in transcriptional and

post-transcriptional regulation of gene expression. They are encoded by specific miRNA genes (Ross et al., 2007) and can degrade mRNA but are synthesised from an unmatched segment of RNA precursor featuring a hairpin structure, thereby inhibiting mRNA translation. Both siRNAs and miRNAs are produced by Dicer-mediated cleavage of longer dsRNA precursors (Figure 1.9), however, miRNAs are endogenous, whereas siRNAs may be endogenously or exogenously derived (Ross et al., 2007). For this thesis, synthetic siRNAs were utilised due to their ability to bind to specific mRNAs and label them for destruction by endonucleases therefore inducing gene silencing.

In humans, siRNAs were first used to target firefly reporter genes resulting in gene specific, siRNA mediated, inhibition, in human HeLa cells (Harborth et al., 2001). Gene silencing can be achieved via transfection of human cells utilising such siRNAs to target a specific gene, frequently using a lipid-based 'transfection' reagent, which is then taken up by mammalian cells, and follows the sequence of events shown in Figure 1.8. This approach offers us the ability to determine gene function and identify possible gene targets which would allow the development of specific therapeutics (Jackson and Linsley, 2010). To mimic this process, synthetic pools of siRNA duplexes have been created, which are the same length as naturally occurring siRNAs and are now commercially available; therefore do not require processing through DICER. Off-target effects have been widely reported when using RNAi, and are defined as the unwanted down-regulation of non-target mRNA. In order to avoid the possibility of these occurring, commercially available siRNAs are designed using bioinformatics software such as BLAST, and pooling of siRNAs to allow for high

transfection efficiency at a lower concentration (ThermoScientific, 2013), as well as chemical modifications, all of which will be discussed further in chapter 3.

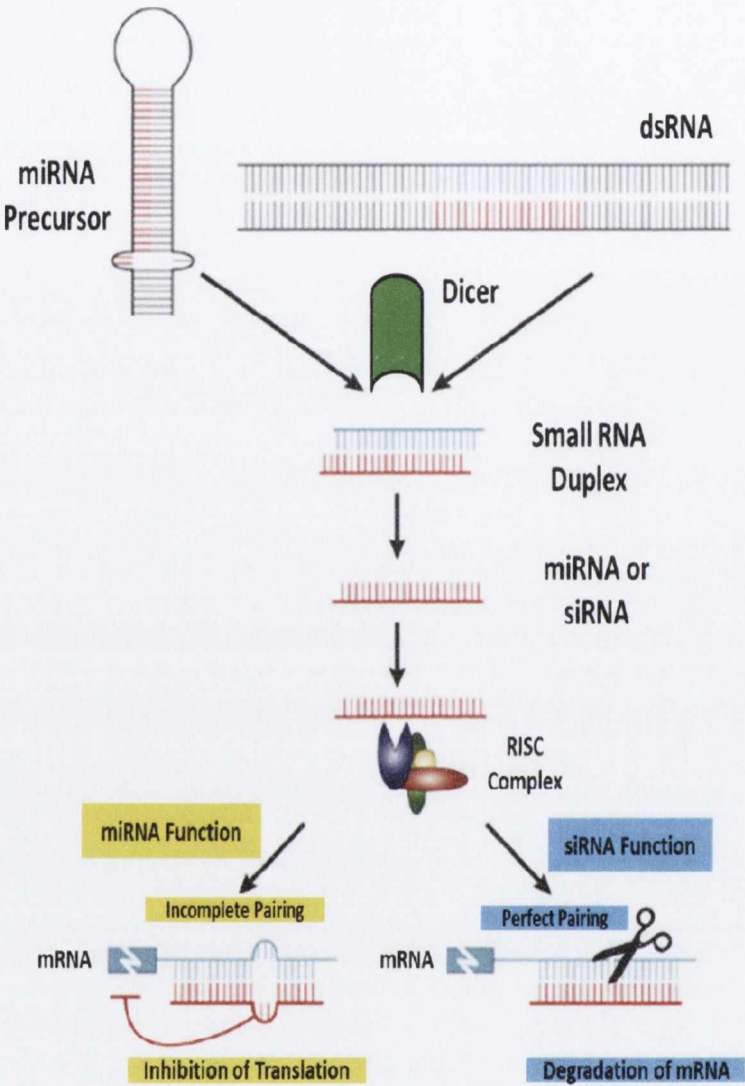


Figure 1.9: Gene Silencing by miRNA and siRNA. miRNA and siRNA use the same mechanism to direct silencing utilising the RISC complex, but miRNAs use incomplete pairing to inhibit translation, while siRNAs use perfect pairing to degrade mRNA.

1.6.1 The use of siRNA Libraries

High-throughput technologies such as large-scale siRNA library screens now play a central role in molecular biology and drug discovery. The availability of libraries of

siRNA pools has contributed greatly to gene function research allowing for the simultaneous analysis of thousands of genes, with the intention of identifying specific functions involved in a specific disease or pathway, with the potential to lead to development of novel drug targets. Dharmacon is one company that developed SMART-pool siRNA libraries, arrayed to target specific 'genomes' or receptor families including the whole human genome, the human druggable genome, the kinase family members and the G-protein coupled receptors (GPCR).

The success of using large scale siRNA library screens to identify genes involved in different cancer types has been widely demonstrated. For example, a kinase family genome RNAi screen was undertaken to identify kinases that modulate triple-negative breast cancer (TNBC) cell growth. The pilot screen was performed in one cell line, SUM149, and confirmatory screens utilised four different breast cancer cells lines, SUM149, MDA-MB-231, BT474-M1 and HR5. Apoptosis was determined by nuclear morphology and Hoechst dye intensity utilising a high-content screening system and results indicated that inhibiting PLK1 with its specific siRNA, blocked growth of TNBC cells by more than 80% in the SUM149 cell line (Hu et al., 2012). A more recent study utilised a lentiviral vector-mediated RNAi screen to investigate the functions of histone acetyltransferases (HAT) on lung cancer cell viability. The end-point assay was the CellTiter One solution cell proliferation assay and results indicate that KAT8 inhibition significantly reduced lung cancer (A549) cell viability by 46% compared to the control (Zhang et al., 2013b).

It is not just within cell lines that RNAi screens have shown success. The first whole human genome RNAi screen *in vivo* identified the role of LIFR as a tumour

suppressor in breast cancer (Iorns et al., 2012). Human mammary epithelial (HMLE) breast cells were transfected with shRNA, then injected into the mammary gland of the mouse, which were sacrificed when the tumour reached 5% of the body weight of the mouse. Genomic DNA was then extracted and used as a template for PCR amplification of the integrated shRNAs using vector derived primers. Not only did this study identify LIFR as a potential tumour suppressor, but it also confirmed the role of TP53 and MNT as tumour suppressors.

Synthetic lethal RNAi screens can provide clues to combinations of therapies that might be particularly useful for specific cancer subtypes. These types of screens combine low concentrations of a drug, generally a chemotherapeutic drug, with whole-genome siRNA screens to sensitise the cancer cells. For example inhibiting NIBP can re-sensitise lapatinib-resistant breast cancer cell lines (Wetterskog et al., 2013) thus highlighting the potential for RNAi mediated screens in the identification of genes involved in cancer development, by inhibiting individual genes or on a synthetic lethal basis to analyse the effect of gene knockdown on drug efficiency.

Within the oesophageal cancer setting, no RNAi library screen has been published thus far, therefore implementation of a large scale siRNA screen in oesophageal cells would be the first of its kind. Although a screen has not been undertaken the efficacy of siRNA mediated gene knockdown has been demonstrated. For example, the effect of STAT3 knockdown by siRNA on cell proliferation was analysed in two oesophageal cell lines, OE21 and OE33, and the effect was determined using the Xcelligance system, which analyses impedance every 15mins for 50hrs. Results indicated a 2-fold decrease in cell proliferation following siRNA STAT3 treatment in

the OE21 cells, and a 2.5-fold decrease in the OE33 cell line (Timme et al., 2013). Another study has highlighted the role of β -catenin in cell cycle arrest and apoptosis, as detected by flow cytometry, indicating that the combination of sodium nitroprusside, a nitric oxide donor which has been identified as a promising agent for cancer treatment, and β -catenin siRNA increased OSCC cell numbers in G₀/G₁ phase. This suggests that down-regulation of β -catenin may increase sensitivity of the OSCC cells to sodium nitroprusside (Wang et al., 2010b). These studies demonstrate that harnessing RNAi technology within the oesophageal cancer research field could prove invaluable for the development of future therapies.

1.6.2 Therapeutic Potential of siRNA Technology

The potential benefits of RNAi based therapy for cancer treatment could include its specificity and high efficiency rates for down-regulating gene expression, and the use of combination therapy to target multiple pathways is thought to be the best approach (Miele et al., 2012), particularly as we learn more about cancer driver mutations. A major draw-back of RNAi treatment is the mode of delivery *in vivo*, as it is necessary for sufficient quantities of the siRNA to reach and target the cytoplasm of the target cell to perform its inhibitory effects. There are many potential approaches to delivery available including liposomal delivery, nanoparticle based delivery and vector-based delivery, but the systemic delivery of siRNA to a target organ remains a major challenge. In relation to oesophageal cancer, this could pose less of a problem, as topical access to the oesophagus is much easier compared to delivery to an internal organ such as the pancreas. Currently there are clinical trials in progress analysing nano-particle mediated delivery of siRNAs in numerous cancer types including, metastatic melanoma, head and neck squamous cell carcinoma and pancreatic

neuroendocrine tumour (Rao et al., 2013). A recent report has also shown the success of RNAi therapeutic delivery for the treatment of several cancers, by silencing VEGF using a new drug ALN-VSP, resulting in a range of positive outcomes, even complete regression of metastasis in one case in a patient with an endometrial tumour (Tabernero et al., 2013).

1.7 Targets Identified Through siRNA Library Screening

The implementation of the siRNA library screen within this thesis identified two immune related genes, leukaemia inhibitory factor (LIF) and complement component 1, subcomponent Q, chain A (C1QA) as genes of interest within the oesophageal cancer setting, by significantly reducing oesophageal cell viability. Both genes are upregulated in BO and OAC, thus they were both further validated in relation to two distinct inflammatory pathways, the STAT3 and Wnt/ β -catenin pathways respectively (Zhang et al., 2011, Jarnicki et al., 2010, Naito et al., 2012, Keerthivasan et al., 2014). Subsequently, a synergy experiment was performed between both LIF; and C1QA, and a panel of 56 immune related genes, to further reveal any possible gene-gene interactions between thus far unrelated immune factors, in their contribution to OAC cell survival. This highlighted possible associations with Runt-related transcription factor 2 (RUNX2), triggering receptor expressed on myeloid cells 2 (TREM2), and pre T-cell antigen receptor alpha (PTCRA) in particular, which are all located in the immune related MHC chromosomal region at 6p21. For this reason, these genes, and the pathways in which they are involved, will now be outlined.

1.7.1 Leukaemia Inhibitory Factor (LIF)

Leukaemia Inhibitory Factor (LIF) is a secreted cytokine of the IL-6 family that is involved in development, infection and inflammation. LIF shares a receptor, gp130, with IL-6, which binds to its receptor and activates JAK kinases, leading to STAT3 phosphorylation and activation (Berishaj et al., 2007), which is known to induce inflammation (Wang et al., 2013c, Silver and Hunter, 2010), and has a known link to oesophageal cancer (Zhang et al., 2011). LIF plays a key role in trophoblast biology, where it controls blastocyst attachment, along with trophoblast invasion, proliferation and differentiation, via the STAT signalling pathway (Suman et al., 2013a).

In cancer biology, less is known about the role that LIF plays. In ovarian cancer high levels of IL-6 and LIF were identified within ascites which can skew monocyte differentiation into tumour-associated macrophage (TAM) like cells, indicating a tumour escape mechanism (Jeannin et al., 2011). Due to its pleiotropic nature, LIF has also been shown to suppress medullary thyroid carcinoma (MTC) cell growth (Arthan et al., 2010, Starenki et al., 2013), while on the other hand it both increases the invasive potential of choriocarcinoma cells and upregulates caspase-4 mRNA indicating that LIF mediated STAT3 activity increases the invasiveness of both trophoblastic and choriocarcinoma cells (Fitzgerald et al., 2005). A similar finding was also noted in malignant myeloma where LIF is important for myeloma progression, as demonstrated by siRNA mediated knockdown of LIF (Kuphal et al., 2013). Although LIF has clearly been studied in depth in relation to stem cells and choriocarcinoma, little or no information can be found in relation to its role in BO and OAC.

1.7.1.1 Therapeutic Benefit of Targeting the STAT3 Pathway

Targeted therapies are now widely used for personal therapy based on molecularly defined cancer types, a successful example of which is the use of Herceptin for the treatment of Her2 positive breast cancer (Pegram et al., 2000). If this technology could be harnessed and directed towards targeting genes within the STAT3 pathway such as IL-6, LIF or STAT3 itself, then it may prove to be of potential importance for the treatment of malignancy.

Transcription factors, such as STAT3, are prime targets for personalised therapy as they coordinate many known oncogenic intracellular pathways. Targeting these pathways has been utilised and successfully implemented by targeting Myc, a transcription factor that is necessary for growth and proliferation of somatic cells, with an inhibitor, Omomyc, to inhibit lung cancer progression in mice (Soucek et al., 2008). Many STAT3 inhibitors that are widely used act by targeting phosphorylation at the Y705 residue which has been deemed necessary for induction of dimerisation leading to nuclear translocation and DNA binding and has been identified in tumour biopsies via immunochemistry. Evidence for the positive effect of inhibiting STAT3 on tumour progression has been identified in several studies; STAT3 knockdown in colitis-associated cancer, skin cancer and large cell lymphomas all resulted in a reduction in cancer progression (Bollrath et al., 2009, Chan et al., 2004, Chiarle et al., 2005).

The main issue with inhibition of STAT3 has been demonstrated in mice, who developed Th1-associated autoimmunity, although it was not systemic autoimmunity, suggesting that it is a specific effect on intrinsic immune surveillance (Kortylewski et

al., 2005). Although there are STAT3 inhibitors commercially available, none have been tested in the BO/OAC setting to date, but based on data that will be presented in this thesis, targeting STAT3 or components of the STAT3 pathway may prove beneficial for BO and OAC treatment.

1.7.2 The C1q and Wnt Signalling Pathway Connection

As previously outlined, the complement system is an integral part of the innate immune response with C1q acting as a recognition signal for immune complexes, triggering activation of the classical complement pathway. C1q deficiency can induce onset of *Systemic lupus erythematosus* (SLE), a long-term autoimmune disorder previously described and is found in $\geq 92\%$ of SLE patients (Pickering and Walport, 2000, Barilla-LaBarca et al., 2013).

Most inflammatory cells express and/or secrete C1q (Ghebrehiwet et al., 2001) and as previously discussed, chronic inflammation such as chronic GORD can play a role in the development BO leading to OAC; thus investigating the role of complement proteins including C1q within this setting may be of interest. There has only been one published paper demonstrating that reduced C1q levels could increase risk of OAC development (Bobryshev et al., 2010). This group previously showed that BO tissue contains antigen-presenting dendritic cells, which can initiate and regulate the immune response (Bobryshev et al., 2009). They used clinical biopsy samples; 15 BO patients, 13 OAC patients and 12 normal oesophageal patient samples and all patients underwent an endoscopy to confirm their status before participation in the study. Both single and double immunostaining was undertaken for C1q, dendritic markers CD83 and DC-SIGN, B-cells, T-cells and macrophages, while RT-PCR was also undertaken

to analyse C1q expression in dendritic cells, and western blot analysis for C1q was analysed in macrophages and dendritic cells. Results showed that normal oesophageal samples (1756 + 228) had higher levels of C1q compared to the BO (1114 + 139) or OAC (1199 + 175) samples, within the extracellular matrix. Co-localisation of extracellular C1q and dendritic cells was also undertaken, indicating that C1q may act as a chemotactic factor. Dendritic cells were found in all samples analysed and *in vitro* studies showed that they produced all three C1q chains, C1qA, C1qB and C1qC while mRNA and protein analysis also showed expression of all three C1q chains in both dendritic cells and macrophages. Overall this study showed that C1q was present within oesophageal tissue samples, with higher expression noted in normal oesophageal samples as compared to BO and OAC.

C1q plays a major role in the recognition of immune complexes, which activates the classical complement pathway as previously described, but more recent research has focused on the role of C1q as a sensor for DAMPs and PAMPs. Most complement proteins are synthesized in the liver, but C1q is different, as it is synthesised extrahepatically by several cell types including epithelial cells and monocytes/macrophages (Colten, 1976, Armbrust et al., 1997). The synthesised molecule can then be secreted into the peri-nuclear region to modulate cell-specific biological responses using autocrine and/or paracrine signalling via cell surface receptors (Ghebrehiwet et al., 2012). This diverse range of functions mediated by C1q may be attributable to the presence of two specific structural features present on the same protein; collagen-like domain (cC1q) and globular-like domain (gC1q), the latter of which helps in the identification of non-self antigens and can help C1q bind to cell surface molecules (Paidassi et al., 2011). Both of these domains have specific

receptors; cC1qR and gC1qR respectively with the binding site of gC1qR on C1 being located on the A-chain (C1QA) of C1q (Ghebrehiwet et al., 2001). In foetal studies, it has been identified that C1q actively promotes trophoblast invasion to the decidua, which can be mediated by gC1qR, by promoting trophoblast adhesion and migration by activation ERK1/2 (Agostinis et al., 2010). Following this it was shown that C1q null mice had a reduced foetal weight, smaller litter size and increased foetal resorption as compared to wild-type (Singh et al., 2011), suggesting that inhibition of locally secreted C1q can affect autocrine/paracrine signalling resulting in pregnancy disorders. During the inflammatory response, activated cells, such as endothelial cells, can overexpress, secrete and/or release soluble gC1qR (sgC1qR), which in turn, binds to cells in an autocrine or paracrine manner (Peterson et al., 1997). Immunohistochemical (IHC) staining of normal and adenocarcinoma oesophageal tissues for gC1qR, showed a significant increase in staining in the OAC samples as compared to the normal samples (Rubinstein et al., 2004) indicating that gC1qR could be beneficial as a tumour cell marker for OAC or may even have therapeutic benefits as a drug target for the treatment of OAC.

The role of complement in cancer is an emerging area of interest with studies showing that cancer cells can either secrete complement inhibitors (C1INH and Factor H) or express membrane complement regulatory proteins (mCRPs) (CD59, CD46 and CD55) (Gancz and Fishelson, 2009, Kolev et al., 2011, Markiewski and Lambris, 2009) to help evade recognition as non-self cells. The complement protein C3a has an anti-tumorigenic effect which may be due to down-regulation of IL-1 β , IL-6 and other pro-inflammatory cytokines (Rutkowski et al., 2010), while excessive amounts of complement proteins C1q and C3 have been found in tumour cells derived from

ascitic fluid samples obtained from ovarian cancer patients. As previously outlined, autocrine and paracrine signalling is highly important in the role of complement in carcinogenesis. A recent study has shown that C1q can sustain the activation of WOX1 tumour suppressor in prostate cancer cells (Hong et al., 2009) and also identified that inhibition of C1q could enhance prostate hyperplasia and tumour formation due to the decrease in WOX1 activation. gC1qR expression has been implicated in enhanced tumour growth and progression with an increase in gC1qR expression linked to a number of cancers, including oesophageal, as outlined previously (Rubinstein et al., 2004). In lung cancer, gC1qR can regulate lamellopodia formation and metastasis (Kim et al., 2011) as identified by gC1qR knockdown, which inhibited tumour formation and metastatic activity. Given these studies, autocrine/paracrine signalling appears to be highly important for tumour formation.

Due to the known link between Wnt signalling, BO and OAC as previously outlined (section 1.5.2.2.3), a recent paper indicating a possible link between C1q and Wnt was of great interest (Naito et al., 2012). This study showed that C1q could activate β -catenin in aging cells, which was demonstrated by treating Hek293 cells with C1q from serum resulting in significantly increased levels of β -catenin. A luciferase assay for the detection of Wnt signalling (TOPFLASH) confirmed that this increase was due to an increase in Wnt signalling, concluding that C1q activates the canonical Wnt pathway by binding to Frizzled receptors, which resulted in C1s-dependent cleavage of LRP6. This is the first time that a link has been noted between the complement C1q and the Wnt pathway, indicating a possible new area of study for identification of novel therapeutic targets. It was thus concluded that C1q activates Wnt signalling,

which has previously been suggested as a possible therapy target for the treatment of oesophageal cancer (Clément et al., 2007, Bobryshev et al., 2010).

1.7.2.1 Complement Component 1, Q Subcomponent, A Chain (C1QA)

C1qA is a constituent of the C1q subcomponent of the complement system. It binds to the B (C1QB) and C (C1QC) chains of C1q, thereby forming C1q (Figure 1.5). C1QA has not been extensively studied in relation to cancer but a genome wide study in 2006 identified that C1QA had an association with ER-negative breast cancer prognosis, indicating that overexpression of C1QA resulted in a better prognosis (Teschendorff et al., 2006), but a follow-up study by the same team found that there was not a link between genetic variation within C1QA and survival of breast cancer patients (Azzato et al., 2010). A specific polymorphism identified in C1QA, C1QA₂₇₆, has been investigated in relation to a number of diseases including lymphoma and breast cancer. It is suggested to be a potential marker for predicted response to R-CHOP, a cocktail of chemotherapy agents including rituximab, in lymphoma patients (Jin et al., 2012), while in follicular lymphoma, this polymorphism has been identified as mediating both the clinical response and duration of response to rituximab (Racila et al., 2008). In breast cancer an association has been identified between this C1QA polymorphism and reduced haematogenous spread of breast cancer (Racila et al., 2006). Although this polymorphism has been identified in lymphoma and breast cancer, it has not been widely studied in other malignancies; therefore it may be of interest to further investigate if it plays a role in the oesophageal carcinogenic sequence.

Although the C1QA₂₇₆ polymorphism appears to play a role in carcinogenesis, over-expression of C1QA has also been investigated. For example, in castration resistant prostate cancer, over-expression of C1QA was found in enriched monocytes in patients with increased risk of mortality indicating a role for macrophage differentiation in CRP cancer (Ross et al., 2012). Therefore a link has been identified between C1QA and cancer, although the studies generally have not been confirmed and the mechanisms by which C1QA affects cancer have not been identified thus far. Another important study showed that C1QA is highly expressed in macrophages including THP1 cells, where it interacts with VISA and that C1QA expression in Hek293T cells increased the RIG-1-mediated antiviral response (Wang et al., 2012b). Although this study is not cancer cell specific, it does confirm that C1QA plays a critical role in the innate immune response. There is currently no published literature for the role of C1QA in BO and OAC.

1.7.2.2 Therapeutic Benefits of Targeting the Complement System and the Wnt Signalling Pathway

Research into the complement system in cancer has been mainly directed towards improving the efficacy of anticancer treatments, but recently the idea of complement inhibition as a therapy itself has come into the limelight. In 2007 the first complement specific drug, eculizumab, an antibody against C5, was approved by the US FDA for the treatment of haemolytic uremic syndrome. It acts by binding to C5 thereby inhibiting the cleavage of C5a and subsequently the formation of the MAC (Ricklin and Lambris, 2013b). Table 1.2 highlights common complement therapeutic drugs, which are currently being used for the treatment of different diseases.

Complement component 1 inhibitor (C1INH) is a heat-labile factor that inhibits esterase function of the first complement component (C1), by inhibiting formation of the C1 component, thus preventing binding of C1r and C1s. Within the body, irregular levels of C1INH can result in hereditary angioedema (HAE), which affects the blood vessels and results in swelling of the skin or the gastrointestinal tract mucosa. Although, as a therapeutic, it has been shown to suppress inflammation by inhibiting complement and kallikrein-kinin systems, and it has been widely studied in sepsis, endotoxic shock, pneumococcal meningitis, malaria, ischemia-reperfusion injury and transplantation (Davis et al., 2010, Davis et al., 2008). Compstatin inhibits C3 cleavage thus blocking activation and amplification, which may be of particular interest in cancer therapy (Janssen et al., 2007). PMX-53 neutralises the C5a/C5aR interaction, therefore blocking the formation of the MAC, which again could prove beneficial in cancer therapy, especially in lung cancer as it has been shown that, when C5aR was blocked, it reduced NSCLC tumour growth (Corrales et al., 2012).

Table 1.2: Current Complement Therapeutics

Compound Name	Complement Target	Compound Class	Clinical Trials
C1INH	C1r, C1s, MASP1, MASP2	Regulator	HAE, Transplantation, Trauma
Compstatin (POT-4)	C3, C3-convertase	Peptide	AMD
Eculizumab	C5	Ab	* Transplantation, Nephropathy, Injury, AMD, Leukaemia
PMX-53	C5aR	Peptidomimetic	No clinical trials at present

Table adapted from (Ricklin and Lambris, 2013b, Pio et al., 2013)

*Clinical trials are being undertaken in 52 different diseases, only 5 are mentioned above.

1.7.3 Runt-Related Transcription Factor 2 (RUNX2)

Runx2 acts as an osteoblast-specific transcription factor and is located at 6p21.1. During embryonic development it controls bone cell differentiation, while after birth it can control bone matrix deposition, in particular, collagen I (Ducy et al., 1999). RUNX2 plays a role in tumour development within hematopoietic lineages as well as in breast and prostate cancer (Inman and Shore, 2003, Lim et al., 2010). It regulates the expression of genes associated with tumour progression, invasion and metastasis, including osteopontin, collagenases, and FAK/PTK2 (Inman and Shore, 2003, van der Deen et al., 2012, Chua et al., 2009), as well as playing a role in angiogenesis by inducing VEGF expression following its interaction with HIF-1 α (Kwon et al., 2011). This study also identified that RUNX2 and HIF-1 α physically interact in the nucleus of osteoblasts and on the chromatin of the VEGF gene, therefore regulating angiogenesis. RUNX2 also plays a role in melanoma, for example, TGF- β can up-regulate RUNX2 expression in 1205LU melanoma cells (Mohammad et al., 2011), which was later confirmed in 6 other melanoma cell lines (Boregowda et al., 2014), while inhibition of RUNX2 can also significantly decrease melanoma cell proliferation, as well as reducing migration (Boregowda et al., 2014). All of these studies highlight the significant role that RUNX2 plays in tumour development and progression; therefore it is not surprising that this gene would be of interest in the oesophageal setting.

Although there is a demonstrated link between oesophageal cancer and RUNX3, little is known about the role of RUNX2 in this setting. RUNX3 is not expressed in the Seg-1 OAC cell line, but it is detected in normal oesophageal mucosa. Therefore it may be necessary for the antiproliferic and apoptotic effects induced by TGF- β within

the Seg-1 cells, following cellular transfection with RUNX3 cDNA (Torquati et al., 2004). Given this information, further elucidation of the role of RUNX2 in OAC may be of interest.

1.7.4 Triggering Receptor Expressed on Myeloid Cells 2 (TREM2)

TREM2, located at 6p21.1, is a cell surface receptor, which is mainly expressed on myeloid cells and has been detected on the surface of immune related cells including dendritic, macrophages and osteoclasts (Bouchon et al., 2000, Turnbull et al., 2006, Cella et al., 2003). The encoded protein plays a role in the immune response and may also be involved in chronic inflammation by triggering the production of constitutive inflammatory cytokines. This was identified by the finding that type II inflammation induces TREM2, both *in vivo* during allergen-induced pulmonary inflammation and *ex vivo* by treating resident peritoneal cells with IL-4 (Turnbull et al., 2006). This study also showed that TREM-2^{-/-} macrophages produced higher levels of inflammatory cytokines when exposed to TLR agonists. A more recent study identified the role of TREM2 in inflammatory bowel disease (IBD), where it is up-regulated in lamina propria dendritic cells (Correale et al., 2013). Inhibition of TREM-2 can also protect against colitis by decreased secretion of proinflammatory cytokines IL-1 β and IL-6, and expression of matrix metalloproteinases, MMP3 and MMP9 (Correale et al., 2013) concluding that TREM2 may act by increasing inflammation within the gut microenvironment.

1.7.5 Pre-T-cell Antigen Receptor Alpha (PTCRA)

PTCRA, located at 6p21.3, regulates T-cell development, but little has been documented about its role in cancer development, although it has been shown that it plays a key role in inflammation. Within the STAT pathway, one study showed that PTCRA is not necessary for STAT5b mediated lymphomagenesis in mice, and that, upon PTCRA inhibition, lymphoma occurred more quickly (Bessette et al., 2008). An association has also been noted between Notch3 and PTRCA in T-cell leukaemia, which is of interest as Notch3 has been shown to be critical for T-cell development, and its constitutive activation can lead to T-cell leukaemia (Campese et al., 2003, Bellavia et al., 2002). A more recent study has shown that Notch3 and PTCRA physically interact, thus this direct relationship could contribute to the maintenance of the constitutive activation of both Notch and pre T-cell receptor signalling pathways in thymocytes (Checquolo et al., 2010). Although little is known about the role of PTCRA in solid tumours, further investigation within the oesophageal setting may shed light on a new role.

1.8 Aims

Oesophageal cancer is currently the 8th most common cancer in the Western world and is on the increase, with the highest incidence noted in males. Current treatments include surgery and chemoradiotherapy which are highly invasive and unspecific, but the identification of small interfering RNAs (siRNA) has opened a new area of molecular biology with the prospect of new cancer drug treatments. The identification of genes whose inhibition via siRNA treatment reduced oesophageal cancer cell proliferation significantly could result in development of more specific cancer treatments. Thus, would siRNA library screening of the druggable genome be able to identify novel genes and associated pathways that impact on oesophageal cell viability? To help answer this question, the aims of this thesis are to:

- Optimize siRNA technology and develop a screening platform for identification of genes involved in cell proliferation in an oesophageal cancer cell model
- Implementation of a large scale druggable genome siRNA library screen, utilising 6022 siRNA gene targets to analyse the effect of knockdown of these genes on oesophageal cell proliferation, as detected via MTT assay
- Statistically analyse the results of the screen using R software platform, utilising two specific packages, CellHTS2 and RNAiR, to identify 'high confidence hits'
- Utilise bioinformatics software IPA and DAVID to ascertain genes that are functionally important
- Validate and characterise hits identified with respect to their function in oesophageal cancer cell survival and proliferation

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Cell Culture

Cell lines representing normal oesophageal, Barrett's oesophagus, oesophageal adenocarcinoma and oesophageal squamous cell carcinoma are described in table 2.1.

Table 2.1: Information on all Cell Lines used Throughout this Project

Cell Line	Origin	Media	Supplements	Supplier	*Optimal Seeding Density	Transfection Reagent (DharmaFECT)
EPC-2	hTERT Immortalised Human oesophageal Keratinocytes	KSFM	PSG; BPE; EGF	Gibco	10x10 ³	4
HET-1A	Normal oesophageal	BEBM	BEGM Supplement Kit	Lonza	7.5x10 ³	2
GOhTRT	Oesophageal Dysplastic	BEBM	BEGM Supplement Kit; 5% FCS	Lonza	5x10 ³	4
SK-GT-4	Oesophageal Adenocarcinoma	RPMI 1640	10% FCS; PSG	Gibco	5x10 ³	4
OE-33	Adenocarcinoma of Lower Oesophagus	RPMI 1640	10% FCS; PSG	Gibco	7.5x10 ³	3
TE-13	Oesophageal Squamous Cell Carcinoma	RPMI 1640	10% FCS; PSG	Gibco	5x10 ³	4
THP-1 [#]	Acute Leukaemia Monocyte	RPMI 1640	10% FCS; PSG	Gibco	-	-
HEK293T [#]	Kidney	DMEM	10% FCS; PSG	Gibco	-	-
HepG2 [#]	Liver Hepatocellular Carcinoma	DMEM	10% FCS; PSG	Gibco	-	-

*Cells per well in a 96-well plate for optimal siRNA transfection

Indicates cell lines that were used as controls in western blots only. These cell lines were not transfected.

Conditions for all cell culture required incubation at 37°C with 5% CO₂. Cells were trypsinised at 70% confluency, using 10% trypsin in Hank's balanced salt solution (HBSS).

2.1.1 Maintenance of Cells

Cell lines were maintained as outlined in table 2.1. Media was stored at 4°C then heated to 37°C before use. BEGM (Lonza) supplement kits were stored at -20°C, along with aliquots of Penicillin/Streptomycin/L-Glutamine (PSG) (Sigma Aldrich), foetal bovine serum (Gibco) and trypsin-EDTA (Gibco). Cells were fed at 48 hours, and sub-cultured at 72 hours.

2.1.2 Sub-culturing of Cells

All cells lines used were adherent, except for THP-1 cells. For adherent cells, media was removed, the cells were then detached from the culture flask using 1mL 10x trypsin-EDTA (Gibco) in 9mL HBSS (Gibco), resulting in a final concentration of 1x trypsin-EDTA. Cells were incubated with 1x trypsin solution for 5 minutes at 37°C, resulting in detachment of cells. The cell suspension was then placed in a 10mL tube, and centrifuged for 5 minutes at 1,500rpm. The supernatant was removed, the cell pellet was re-suspended in 5mL complete media and cells were re-seeded at the necessary concentration. Media used was cell line dependent.

For THP-1 suspension cell line, trypsinisation is not necessary. The cell suspension was placed into a 25mL tube and centrifuged for 5 minutes at 1,500rpm. The supernatant was then removed, and the cell pellet was re-suspended in complete RPMI 1640 media. Cells were re-seeded at the necessary concentration.

2.1.3 Long Term Storage and Resuscitation of Cell Lines

Cell stocks were stored by cryopreservation. The cells are pelleted as per section 2.1.2, the pellet was then re-suspended in foetal bovine serum (FBS) (Gibco) containing 10% dimethyl Sulphoxide (DMSO) (Sigma-Aldrich). Cryovials were then filled with 1mL of this cell suspension and stored at -80°C. For long term storage, cryovials were transferred to liquid nitrogen. For resuscitation, the cells were rapidly defrosted, and placed in pre-warmed (37°C) media containing 20% FCS. Cells were allowed to adhere. At 24 hours, media was removed to remove any DMSO, fresh media was then added. Cells were then sub-cultured as per section 2.1.2.

2.1.4 Cell Counting

Each cell line differs in growth rate, therefore requiring different seeding densities for experiments. Cells were counted using a haemocytometer (Fig 2.1). A cover slip was placed over a clean haemocytometer then 10µL cell suspension was added under the cover slip. Cells contained within the four red boxes highlighted in figure 2.1 were counted. The total was divided by 4 to get the average per box. This was then multiplied by 10 to estimate the amount of cells per microliter. A cell suspension containing the correct number of cells was then prepared using this estimated value.

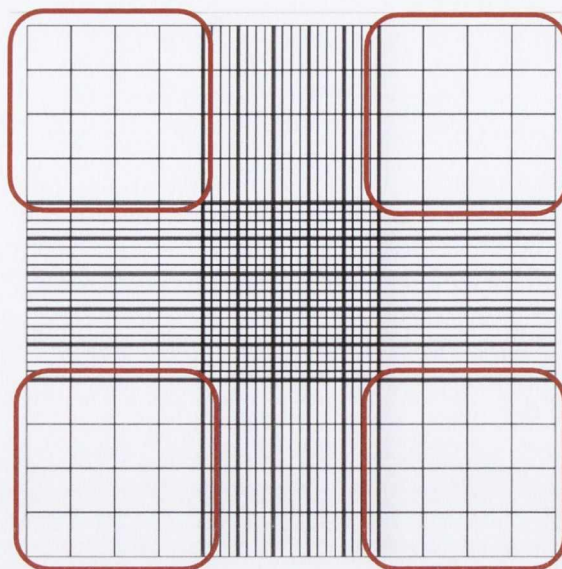


Figure 2.1: Haemocytometer Grid to Facilitate the Calculation of Cell Number. Red boxes indicate the areas of the haemocytometer used to estimate cell count. Cell number from all four boxes was added, then divided by 4 to get the average.

2.2 Dharmacon Human Druggable Genome siRNA Library

The Human siGENOME Druggable Genome Library contains the genes that are considered potential therapeutic targets, which include kinases, proteases, phosphatases, GPCRs, ion channels, ubiquitin ligases, and genes involved in senescence, autophagy, DNA repair, and characterized nuclear receptors (Thermo Scientific). The library was designed utilising SMARTpool technology in which each gene target contains a mixture of 4 siRNA sequences provided as a single reagent. The human druggable genome siRNA library contains 76 plates, with each well of the plate containing a siRNA pool targeting an individual gene, arrayed in a 96-well format.

2.3 Delivery of siRNA to Cells

Small interfering RNAs (siGENOME-SMARTpool; Dharmacon) were transfected into cells (Fig 2.2) using the lipid based transfection reagent, DharmaFECT (Dharmacon). The siRNA and transfection reagent were diluted to concentrations as indicated in individual experiments, using media without supplements, and incubated at room temperature for 5 minutes. The solutions were then combined and allowed complex for 20 minutes at room temperature. Forward transfection involves pre-plating the cells for 24 hours before addition of the siRNA and transfection reagent complex. This approach is optimal for smaller experiments, but not so for large scale high-throughput strategies where setup time should be kept to a minimum. Given this, we seeded the cells before addition of the siRNA/transfection reagent complex, but did not allow them to adhere. This method was implemented for the screen and transfection involved the addition of 80µL cell suspension, at the optimised seeding density being added to each well, then 20µL of the siRNA/transfection reagent complex being added to this. Plates were then incubated for 96 hours at 37°C with 5% CO₂.

A 'scrambled' non-targeting siRNA (Dharmacon) was utilised as the negative control and siTOX (Dharmacon) delivery, transfection and effect positive controls. It is important for distinguishing sequence-specific silencing from non-specific effects in the RNAi experiment while the positive control siCONTROL TOX, is a cytotoxic siRNA sequence that induces cell death. siRNAs targeting GATA6 and Bcl-xL were used as definitive positive controls in cell growth and death assays, as they had previously been shown in the laboratory to affect cell death and target known functional genes.

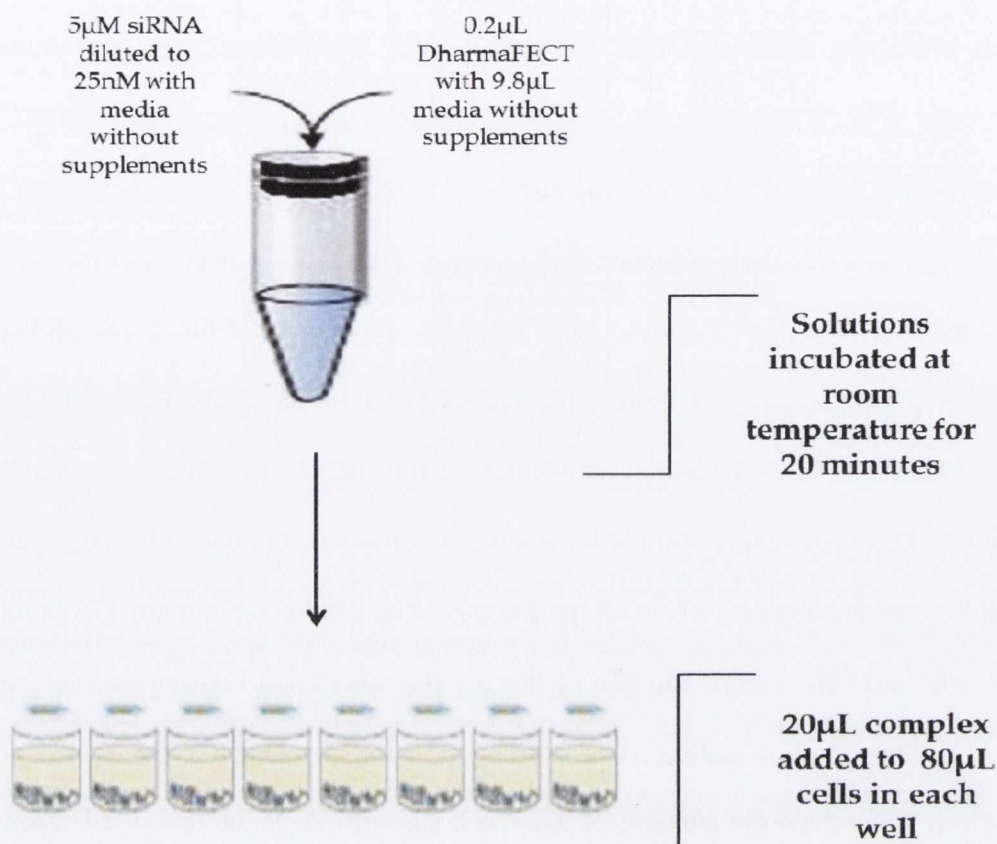


Figure 2.2: Reverse Transfection siRNA Delivery to Cells. The siRNA and transfection reagent are incubated for 20 minutes to form the siRNA/transfection reagent complex. The cells are seeded in the plates during this time. The complex is then added to the corresponding wells. Plates are then incubated for time indicated in individual experiments, at 37°C with 5% CO₂

2.4 Cell Proliferation and Viability Assay

Measurements of cell viability were necessary after treatment of cells with the siRNA, therefore the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay was carried out, as described by (Mosmann, 1983). A kit supplied by ATCC was utilised, containing both MTT and detergent reagent. At 93 hours post-

transfection, 10 μ L MTT was added to each well using a multichannel repetman (Thermo Scientific) then incubated for 3 hours at 37°C. After 3 hours, 100 μ L Detergent Reagent was added using a multichannel repetman (Rainin). Plates were then covered with tin foil to protect them from light, and left at room temperature overnight. Cell viability was calculated by reading the absorbance at 570nm using a spectrophotometer.

2.5 Pilot Reverse Transfection siRNA Screening

Optimal plate layout, positive and negative control performance were initially tested in pilot screening approaches. A *library plate* is the plate which contains the siRNAs at a concentration of 20 μ M. From this, 1 μ L of the siRNA was taken, and transferred to the corresponding well in a polypropylene v-bottom plate containing 3 μ L x siBuffer. This plate is termed the *daughter plate*, which now contains the siRNA at a concentration of 5 μ M. This plate can either then be frozen until needed, or used immediately.

Figure 2.3 shows; from one *library plate*, 3 *daughter plates* were created. From each daughter plate, 4 transfection plates were created. This constituted one *pilot screen*. Conditions for the transfection were kept constant; GOhTRT cells were seeded at 5x10³ cells/well, at 25nM siRNA with 0.2 μ L DharmaFECT4. Cells were added to all 96-wells in each transfection plate, then (control) siRNAs were added to columns 1 and 2 only (Fig. 2.4). Cells were incubated at 37°C with 5% CO₂, then MTT assay was performed as per section 2.4.

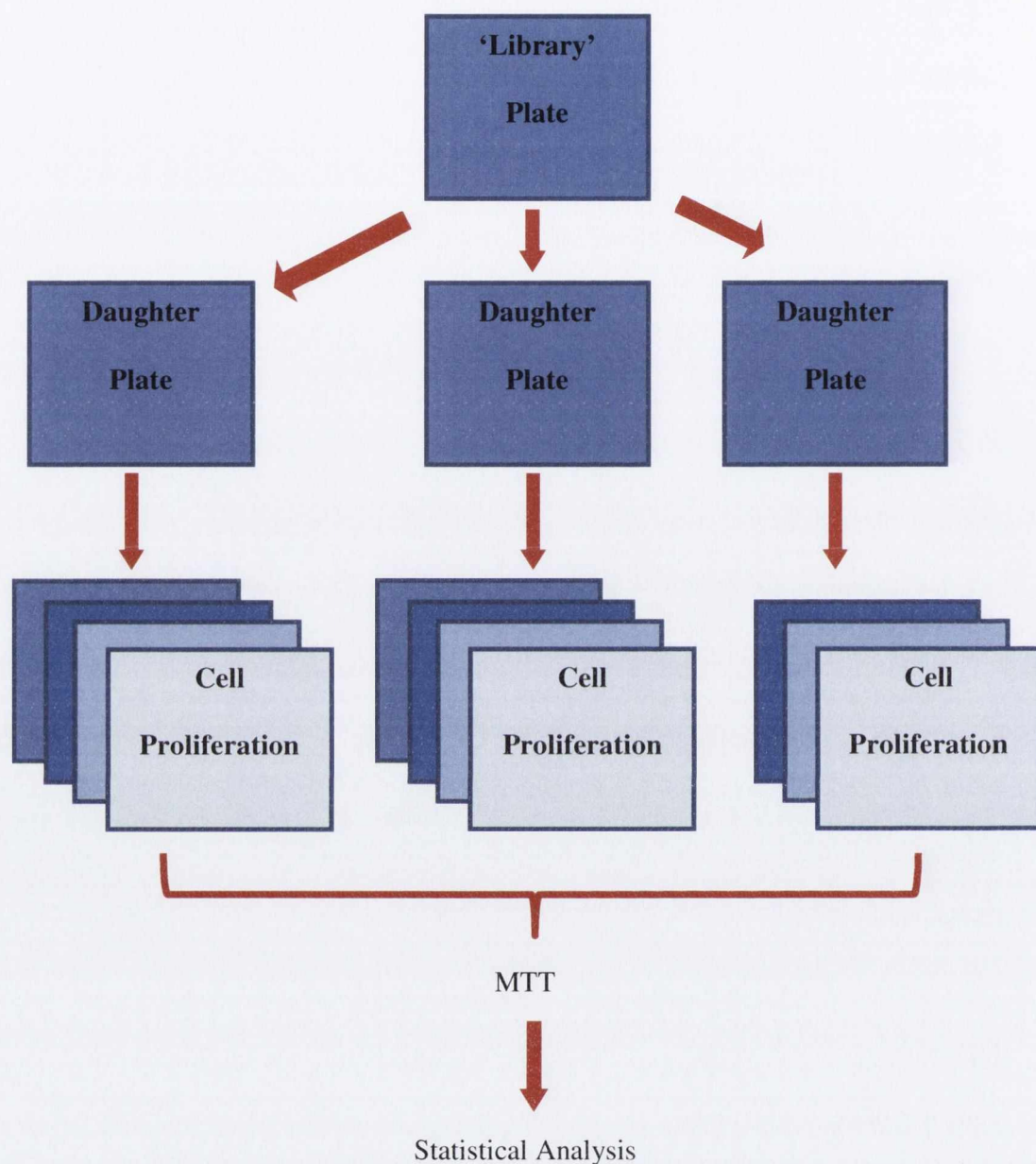


Figure 2.3: Pilot Screen Setup. Pilot screens were setup to analyse plate layout and transfection quality on a larger scale. Three daughter plates resulted in twelve transfection plates. This was later up-scaled to 5 daughter plates and 20 transfection plates. MTT cell viability assay was then performed and quality control statistical analysis was undertaken on resulting data.

2.6 siRNA Library Screening Development and Implementation.

The druggable genome siRNA library contains 6,022 pooled siRNAs arrayed in 96-well format, each targeting the corresponding gene. The druggable genome is comprised of genes that are known to bind drug-like molecules (Hopkins and Groom, 2002). Due to the large number of plates in the library (76), the screen was performed in batches. Figure 2.5 depicts the method by which each of the 76 library plates were reconstituted and aliquoted into a corresponding daughter plate. This smaller number was chosen to reduce the risk of any mistakes or variation throughout. Cells were pre-plated but not allowed to adhere before addition of the siRNA/transfection reagent complex.

As per the method developed in pilot screening experiments of positive and negative controls, *daughter* druggable genome library screening plates containing 1 μ l (20 μ M) of each siRNA per well were prepared from *master* library plates 1-76 in 96 well v-bottomed plates. Positive (siGATA6 and siBCL-xL) and negative (siNon-targeting) siRNA controls were also added to each daughter plate prior to storage or use in screening assays at equal concentrations as per figure 2.4.

From each formatted *daughter* library plate enough siRNA for 8 assay plates at 25nm siRNA per well, was provided. On day of batch screening frozen v-bottom polypropylene 96-well *daughter* plates were thawed and then centrifuged to collect the siRNA prior to addition of 3 μ L 1x siRNA buffer per well and 0.2 μ L DharmaFECT4 transfection reagent per well and incubated for 20 mins. Cells were plated into 96well culture plates (Nunc) as previously outlined, 5x10³ cells/well,

utilising the Wellmate automated media dispenser (Matrix Wellmate). Four transfection plates were prepared in this manor utilising automated pipetting (Fig. 2.5). Three plates would be analysed by MTT cell proliferation assay, and one plate was fixed with 8% PFA and then stained for actin, tubulin and Hoechst for analysis of invasive potential based in morphological changes induced by siRNA inhibition of the specific genes. Transfection reagent and conditions, cell seeding and siRNA concentration were optimised prior to the screen. The use of reverse transfection (Fig 2.2), meant addition of 80µL cells, at a seeding density of 5×10^3 cells/well, to each well using the Matrix Wellmate, then 20µL of the siRNA/transfection reagent complex was added to this, using a multichannel repetman (Thermo Scientific). Plates were then incubated as per section 2.4 before the appropriate assay was performed.

Resting	Resting	1	9	17	25	33	41	49	57	65	73
siNT	siNT	2	10	18	26	34	42	50	58	66	74
siNT	siNT	3	11	19	27	35	43	51	59	67	75
siNT	siNT	4	12	20	28	36	44	52	60	68	76
siBcl-xL	siGATA6	5	13	21	29	37	45	53	61	69	77
siBcl-xL	siGATA6	6	14	22	30	38	46	54	62	70	78
siBcl-xL	siGATA6	7	15	23	31	39	47	55	63	71	79
Resting	Resting	8	16	24	32	40	48	56	64	72	80

Figure 2.4: 96-well Plate Layout of Druggable Genome Library. Columns 1 & 2 are available for placement of controls. Druggable genome siRNAs are pre-plated in columns 3-12. Untreated Cells: grey, siRNA non-targeting: yellow, siRNA Bcl-xL: Green, siRNA GATA6: Blue, Library siRNAs: Pink

During the screen setup, an account was kept of any irregularities noticed. Any abnormalities or infections were noted to allow for confident removal from plate analysis and screen interpretation. While reading the absorbance values following the MTT cell viability assay, a detailed account was also taken of any wells or plates that

contained infections. Again this allowed for removal of these wells from the data analysis.

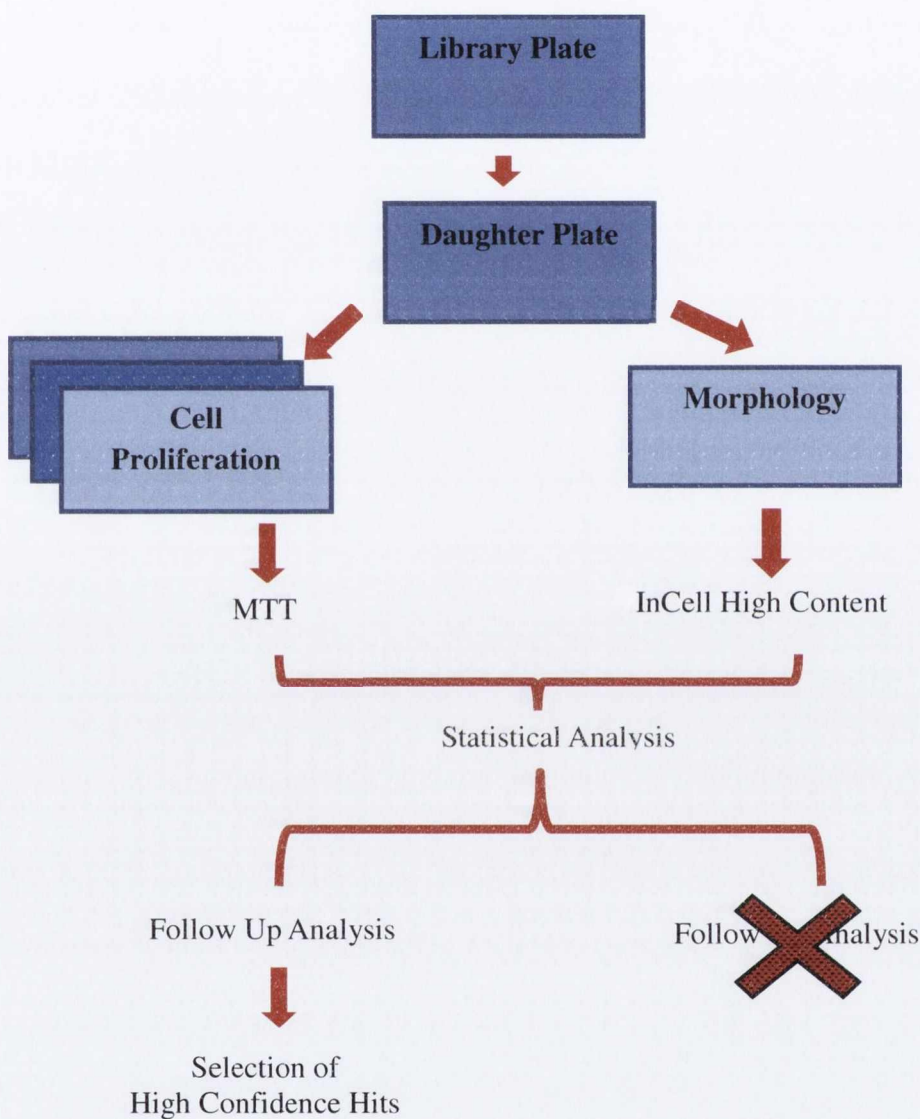


Figure 2.5: Flow-chart of Screen Method. To analyse effect of siRNA gene silencing on cell proliferation, the library plates were reconstituted, aliquoted into daughter plates, the transfection was facilitated in transfection plates for either cell proliferation or morphology analysis, as shown. Statistical analysis was done on all data, while follow up analysis was only carried out on cell proliferation data for this thesis. Hits were selected based specifically on this data.

2.7 Further validation of immune related targets and examination of synergistic interaction

An immune panel, using 56 immune related siRNAs taken from the Druggable Genome siRNA Library was arrayed as per appendix 7.1, while the screen method is shown in figure 2.6. Briefly, one library plate (appendix 7.1) contained 56 immune related siRNAs in the corresponding wells. A daughter plate was setup for each experiment; 1) Immune related genes only, 2) synergy of LIF and immune related genes, 3) synergy of C1QA and immune related genes. From each daughter plate, 6 transfection plates were created; 3 to analyse cell proliferation via MTT assay, and 3 were fixed with 8% PFA for immunofluorescent staining, to analyse cell morphology, at a later stage. In synergistic experiments daughter plates were pre-filled with siRNAs targeting LIF and/or C1QA before addition of the 56 other immune related genes. Transfection reagent and conditions, cell seeding and siRNA concentration, were optimised prior to the screen. The transfection method (Fig 2.2), as previously outlined was utilised in these experiments, briefly 80µL of media, containing cells at a seeding density of 5×10^3 cells/well, was added to each well, 20µL of the siRNA/transfection reagent complex was added to this, as per section 2.3. Plates were then incubated as per section 2.4 before the appropriate assay was performed.

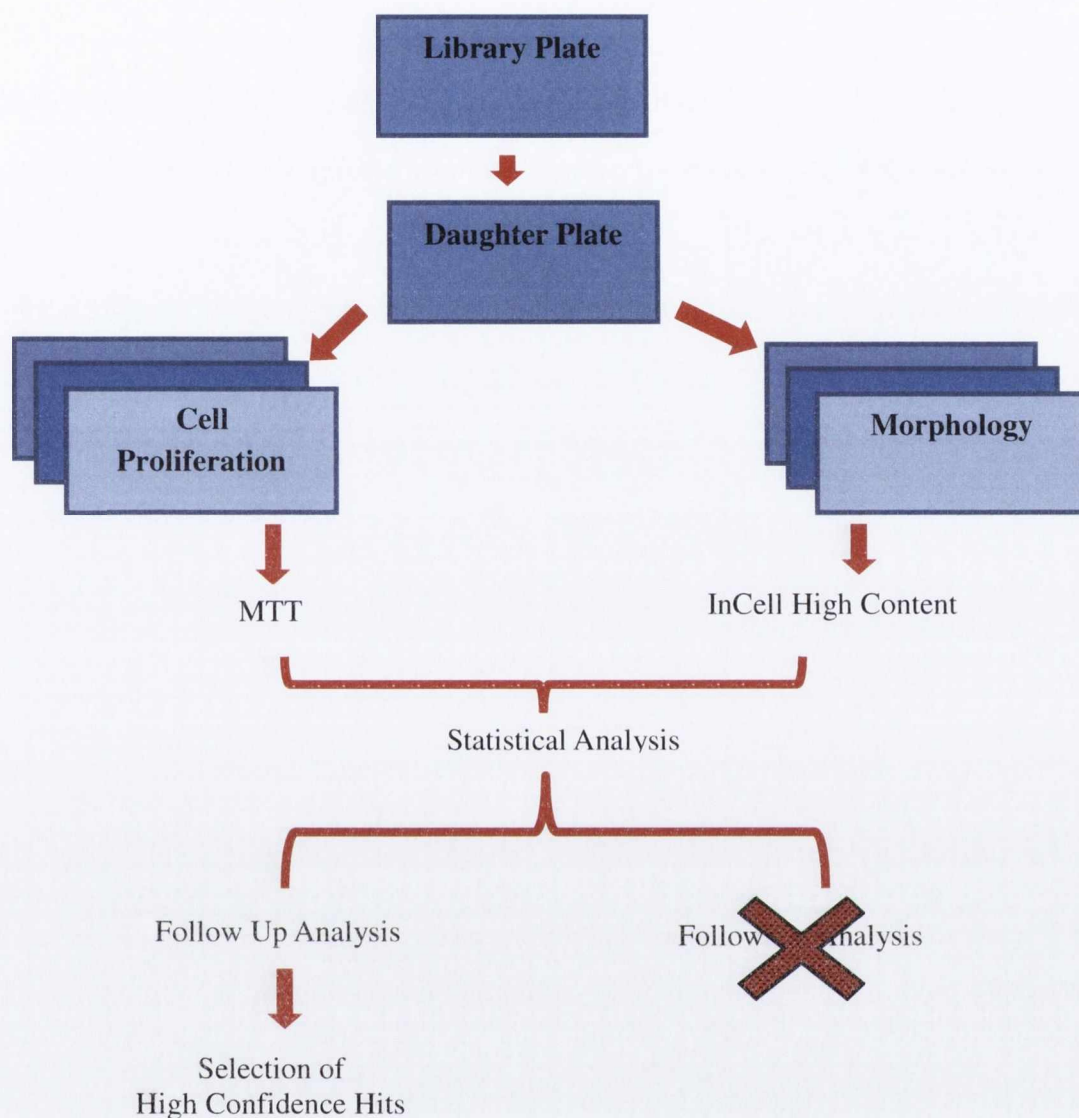


Figure 2.6: Flow Diagram Representing Setup for Synergy Experiments with Immune Panel. To determine the synergistic effect of a double knockdown between LIF and C1QA, and a panel of immune related siRNAs. Corresponding siRNAs from the library plates were aliquoted into daughter plates, and transfection was facilitated in transfection plates for either cell proliferation or morphology analysis, as shown. Statistical and follow-up analysis was performed on cell proliferation data only. Hits were selected based on this data.

2.8 Statistical Analysis

CellHTS2 was first utilised, in conjunction with RNAither for quality control and hit selection. The benefits of RNAither over CellHTS2 include; more flexibility in relation to plate size and number of replicates, as well as more options for methods of normalization, resulting in an output that is easily understandable in HTML format (Rieber et al., 2009). Figure 2.7 shows the statistical tests used, for both quality control and hit selection, throughout. The raw data obtained from our screen was formatted into text files to facilitate the use of RNAither, a package within R statistical software, designed specifically for analysis of large scale RNAi results. A computer script was created (appendix 8.2) to facilitate this. The first step was to generate a dataset or matrix table matching absorbance's or normalised results with genes names and ID's.

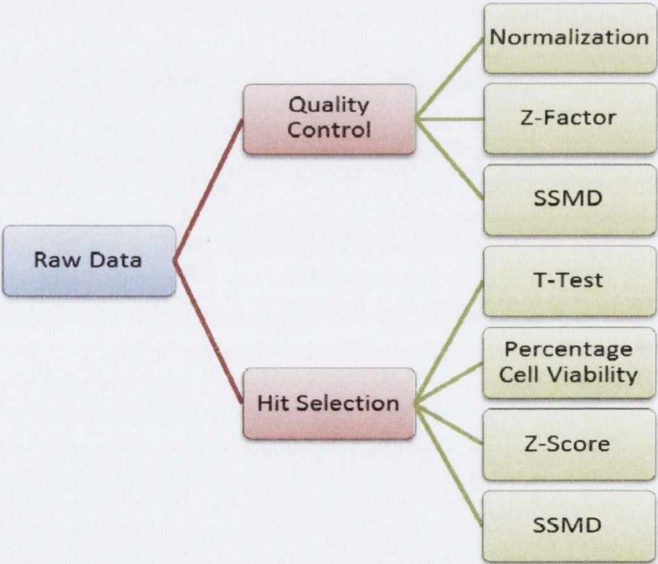


Figure 2.7: Flow Chart of Statistical Analysis Process for Screen. There are two main arms of statistical analysis that must be undertaken, quality control to analyse the quality of the screen, and hit selection to determine the siRNAs that reduce cell viability the most, that should be brought forward for verification, and finally validation. Both arms contain t-test and SSMD, but SSMD formula is different for each (QC and Hit Selection).

2.8.1 Quality Control

Data quality assurance and control (QA/QC) determines if the screen performed optimally using statistical analysis of the performance of the positive and negative siRNA controls. Data was normalized to correct for any differences caused by batch-to-batch variation. Normalisation to the median of the negative controls, per plate, was chosen as it shows consistently similar effect size, and adjusts of spatial effects. Outlier plates and wells were removed as they would skew the data and increase the rate of false positive and negatives. They were identified during the screen setup, before the data was generated. Outliers were removed following normalisation.

The Z-factor is calculated by determining the *separation band*, where dynamic range is the difference between the means of the negative and positive controls. By comparing the dynamic range and the separation band you can assess how well the assay performed. The formula for generating the Z-factor is shown below and in figure 2.8. It is calculated using the mean and standard deviation of both the controls and sample (positive control).

$$Z = 1 - \frac{[3(SD_{\text{sample}}) + 3(SD_{\text{control}})]}{|\text{mean of sample} - \text{mean of control}|}$$

The Strictly Standardised Mean Difference (SSMD) for quality control takes into consideration data variability in both positive and negative controls. It uses the standard deviation of the difference between both the negative and positive controls, whereas the Z-Factor uses the sum of the standard deviation of both controls. The formula used for generating the SSMD ($\hat{\beta}$) for quality control, is shown below and in figure 2.8. It also uses the mean and standard deviation of the negative and positive controls.

$$\hat{\beta} = \frac{\bar{X}_p - \bar{X}_n}{\sqrt{s^2_p + s^2_n}}$$

The t-statistic equation assumes data has a central t-distribution with 'n-1' degrees of freedom, and null hypothesis of zero mean difference. It analyses the mean difference between the positive and negative controls, therefore analysing the significance of the reduction in cell viability. The equation for the t-test uses the difference between the means of the positive and negative controls, the standard deviation of this difference and the samples size/number. Figure 2.8 shows the equations used for each statistical test.

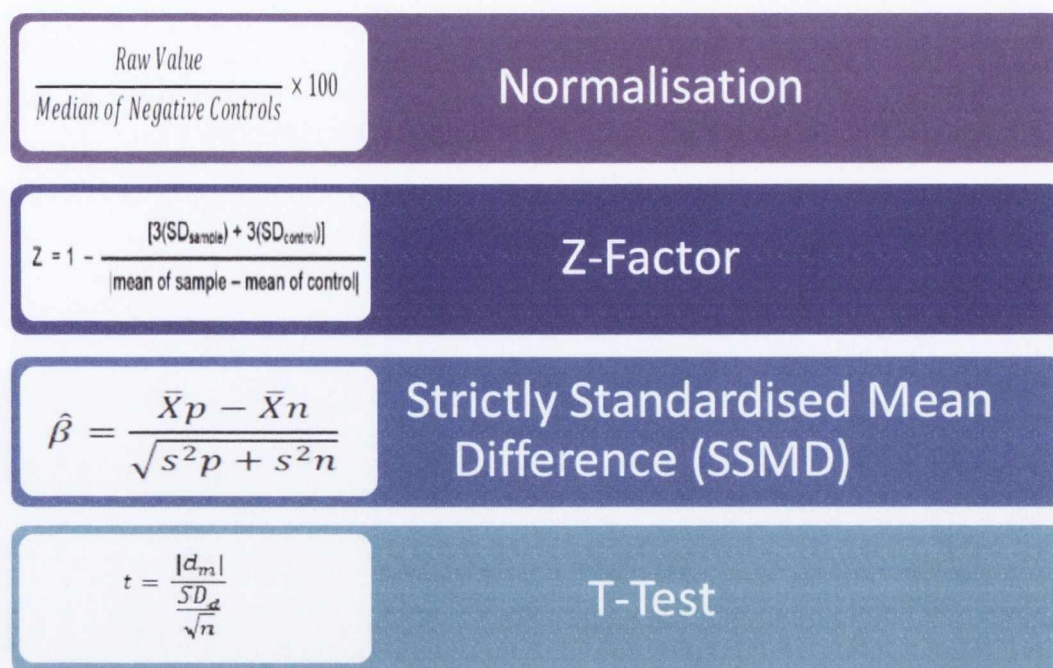


Figure 2.8: Basic Statistics for Quality Control. D_m : Difference between mean of a given siRNA and median of the negative control; SD_d : Standard deviation of a given siRNA; N : Number of replicates of a given siRNA; X_p : Mean of positive control; S_p : Standard deviation of positive control; X_n : Mean of negative control; S_n : Standard deviation of negative control; $\hat{\beta}$: estimated strictly standardized mean difference (SSMD).

2.8.2 Hit Selection

Four statistical tests were utilised for hit selection (Fig. 2.9). Percentage cell viability is the median of triplicate sample wells, represented as a percentage of the median of the negative control (100%), using the formula shown in figure 2.9. The t-test statistic has previously been described in section 2.8.1.

Z-score determines the number of standard deviations a sample is away from the mean. The robust Z-Score uses the median and MAD (median of absolute deviations) which is more robust and insensitive to outliers than mean and standard deviation. It is calculated using the median of each sample, the median of the negative controls and

the MAD. SSMD for ‘hit selection’ uses a different equation to that used for quality control. It is the mean divided by the standard deviation of a difference between two random values each from one of two groups. It is again calculated using the formula in figure 2.9.

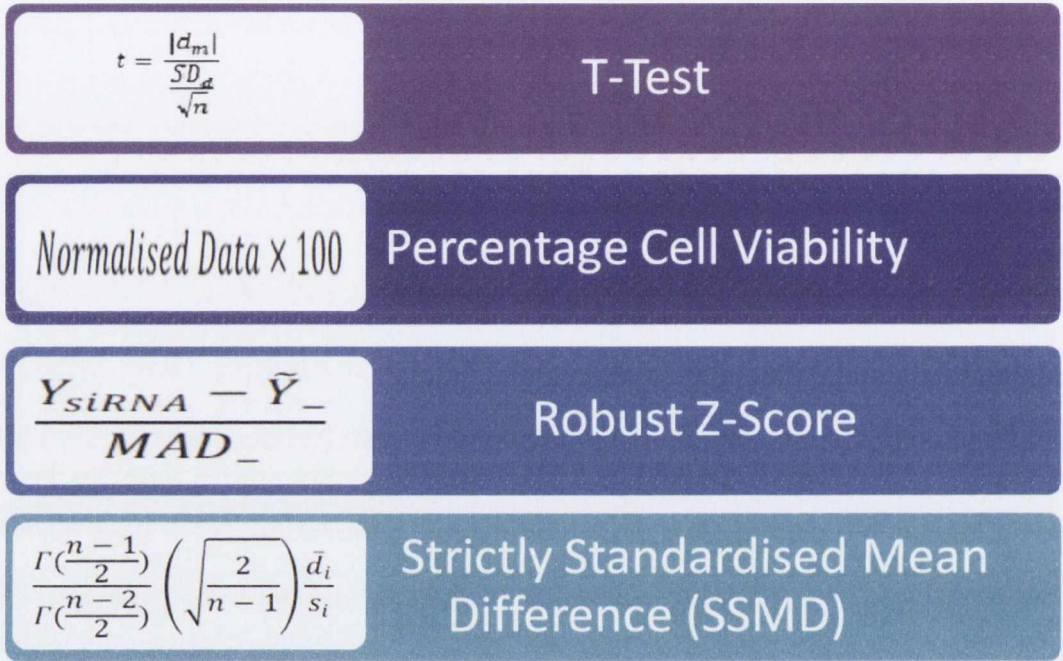


Figure 2.9: Statistical Tests Used for Hit Selection. Γ : gamma function; Y_{siRNA} : measured value of siRNA; $\tilde{Y}_-$: median of negative controls; D_m : Difference between mean of a given siRNA and median of the negative control; SD_d : Standard deviation of a given siRNA; N : Number of replicates of a given siRNA; $\bar{d}_i$: sample mean; s_i : sample standard deviation; **$MAD = 1.4826 \text{ median}(|y_i - \text{median}(y)|)$** , where the constant 1.4826 is chosen to make MAD equivalent to SD when values are normally distributed.

Table 2.2: SSMD-Based Criteria for Hit Selection

Quality	SSMD Hit Selection Criteria
Extremely Weak	$-0.25 \leq SSMD < 0$
Very Weak	$-0.5 \leq SSMD < -0.25$
Weak	$-0.75 < SSMD < -0.5$
Fairly Weak	$-1 < SSMD \leq -0.75$
Fairly Moderate	$-1.28 < SSMD \leq -1$
Moderate	$-1.645 < SSMD \leq -1.28$
Fairly Strong	$-2 < SSMD \leq -1.645$
Strong	$-3 < SSMD \leq -2$
Very Strong	$-5 < SSMD \leq -3$
Extremely Strong	$SSMD \leq -5$

2.8.2.1 Hit Selection Cut-Off Criteria

For data analysis four statistical tests were chosen (Fig 2.9), with stringent cut-offs (Table 2.3). The top genes identified obeyed all of the criteria. A t-test cut off of $P = 0.01$ was utilised to reduce positive findings; cell viability cut-off was set to 40% which is equivalent to the positive control (GATA6); a robust Z-Score cut-off of -1.6 was chosen corresponding to a p-value of 0.05. An SSMD cut-off of -5 was chosen as this is the significance level indicated by (Zhang, 2011b) (Table 2.2).

Table 2.3: Hit Selection Cut-Off Criteria for all Statistical Tests

T-Test	% Cell Viability (CV)	Robust Z-Score	SSMD
<0.01	<40%	< -1.6	< -5

2.9 RNA Extraction

RNA extraction was carried out using the NucleoSpin RNA extraction kit (Macherey-Nagel), as per manufacturer’s instructions. Briefly, the cells were lysed and filtrated using the NucleoSpin Filter. The RNA binding conditions were adjusted using 70% ethanol, then the RNA was bound using the NucleoSpin RNA Column. Following these steps, a membrane desalting buffer was run through the column to allow for a more effective rDNase digest. The silica membrane was washed and the RNA eluted. Following extraction, the RNA was quantified using a NanoDrop Spectrophotometer (NanoDrop Technologies).

2.10 Real-Time Reverse Transcription Polymerase Chain Reaction

RNA was reverse transcribed using the RETROscript kit (Ambion/Applied Biosystems). Briefly, the RNA was reverse transcribed using heat denaturation, in which the a cocktail containing; reverse transcription buffer, dNTP mix, RNase inhibitor and reverse transcriptase, was heated to 42°C for 1 hour before being increased to 92°C for 10 minutes. This increase in temperature deactivates the reverse transcription. The PCR was run using a 384-well plate, with each well containing a

cocktail of cDNA, primer corresponding to the gene of interest, and TaqMan Gene Expression Master Mix. The PCR was run in the ABI Prism 7900T real time cycler (Applied Biosystems), where the cDNA was denatured at 70°C for 3 minutes, then annealed to the primer at 42°C. Fold change was measured using the $\Delta\Delta\text{Ct}$ relative expression method, in which the relative expression of the sample was compared to that of a negative control. In this case, the endogenous control was glyceraldehyde 3-phosphate dehydrogenase (GAPDH), while RNA from resting cells was used as a negative reference from which to determine fold change.

2.11 Detection of Protein Levels in Oesophageal Cancer Cells

2.11.1 Protein Extraction

For protein analysis, the GOhTRT cells were seeded at 350×10^3 cells/well, in triplicate wells of a 6-well plate. A final concentration of 25nM was used for the siRNA transfection, with 5 μ L DharmaFECT4 forming the siRNA/transfection reagent complex as per section 2.3. At 72hrs post-transfection, media was removed from all wells, and replaced with 1mL HBSS. Cells were then scraped from the wells using a pipette tip and the cell suspension was centrifuged at 4°C for 5 minutes to form a pellet. The NE-PER Nuclear and Cytoplasmic protein extraction kit (Thermo Scientific) was used following the manufacturer's instructions, to extract protein from both the cytoplasmic and nuclear fractions separately. Supernatant's was transferred to pre-cooled eppendorfs to be stored at -20°C until needed.

2.11.2 BCA Curve

Protein was quantified using a Micro BCA Protein Assay kit (Thermo Scientific) as per manufacturer's instructions. Standards using known concentrations of bovine serum albumin (BSA) were prepared using ultra-pure water. Following protein extraction, 5 μ L of the protein was diluted to 150 μ L using ultra-pure water. A control was also used which contained the CER and NER reagents only, which would help remove any background signalling that may result from the extraction reagents themselves. A working reagent (WR) supplied within the kit consists of three reagents which are combined in a ratio of 25:24:1. A 96-well plate was then setup, with triplicate wells, using 150 μ L of the protein samples and standards, to which 150 μ L of the WR was added. The plate was sealed and incubated for 2 hours at 37°C. The plate was read using a spectrophotometer at 562nm. The concentrations of the unknown samples were calculated using the standard curve generated from the BSA standards.

2.11.3 Acetone Precipitation

To precipitate the protein from the extraction buffer, the sample was placed in ice-cold acetone at a ratio of 4:1, acetone: sample. The tube containing the sample and acetone was placed at -20°C for 45-60 minutes. The tube was centrifuged at 6,500rpm for 5 minutes at 4°C. The supernatant was removed and the pellet re-suspended in 25 μ L sample buffer (Sigma Aldrich). The re-suspended protein was boiled at 100°C for 5-10 minutes depending on the pellet size.

2.12 Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Protein samples were boiled for 5mins before loading into the gels. At the same time 25µL pre-stained molecular weight marker (Cell Signalling) was heated to 100°C for 5-10 minutes. Resolving and stacking gels were made as per table 2.4. To each lane of the gel, 25µL of the re-suspended was added. All empty lanes were filled with 25µL sample buffer only. Molecular weight marker (15µL) was also added to one lane on each gel. Gels were run at 50mA per gel.

Table 2.4: Reagents Used in Creation of Resolving and Stacking Gels

Reagent	Resolving Gel	Stacking Gel
Distilled Water	7.9mL	3.4mL
Acrylamide	6.7mL	0.83mL
Tris (pH 6.8)	-	0.63mL
Tris (pH 8.8)	5.0mL	-
10% w/v APS	200µL	50µL
10% w/v SDS	200µL	50µL
TEMED	8µL	5µL

TEMED: N, N, N', N'-Tetramethylethylenediamine

2.13 Immunofluorescent Imaging

2.13.1 InCell Imaging

The GE InCell 1000 kinetic assay development machine was utilised for imaging protein localisation on a subcellular level. 5x10³ cells/well were seeded in a 96-well

plate. They were fixed with 4% paraformaldehyde (PFA) at 96-hours post-transfection, and incubated at 37°C for 15 minutes. Wells were then washed with 0.01% PBS-Tween, permeabilised with 0.3% Triton-X for 10 minutes at room temperature (RT), then blocked with 3% bovine serum albumin (BSA) for 1 hour at RT. Primary antibodies were diluted, with 3% BSA, to specific ratio after optimization using dilutions within ranges supplied for specific antibody. Plates were incubated at 4°C overnight on an orbital shaker before addition of species-specific secondary antibody. The secondary antibody was also diluted to specific ratio with 3% BSA then incubated at room temperature, protected from light, for 1 hour. Hoechst 33342 was used at a ratio of 1:5000 as a nuclear stain in all experiments, which was added in parallel to the secondary antibody for 30 minutes, also protected from light. Wells were washed with 0.01% PBS-Tween, and 200µL PBS was left in the wells while the plate was stored at 4°C until needed. Cells treated with secondary antibody only were always prepared in parallel to serve as a negative control for specific binding of the primary and secondary antibodies.

2.13.2 Confocal Microscopy

Sterile circular cover slips were placed in 24-well plates, to which cells were added to adhere to the cover slips. siRNA transfection was carried out as per section 2.3, but increased to correspond with plating in a 24-well plate. At 96 hours post-transfection cells were fixed with 8% PFA, permeabilised and blocked as per section 2.13.1. Primary and secondary antibodies were diluted and added as per section 2.13.1 also in the 24 well plates. Coverslips were then mounted to glass slides using Fluoromount aqueous mounting media (Sigma-Aldrich) before imaging using the Zeiss 510 LSM Meta confocal microscope.

2.14 Recovery Experiment of siRNA Phenotype

In phenotypic restoration experiments one 96-well plate containing 5×10^3 cells/well and 25nM siRNA/transfection reagent complex were added for analysis by MTT assay, one 12-well plate containing 5×10^3 cells/well and 25nM siRNA/transfection reagent complex were added for analysis at an mRNA level via RT-PCR, and one 6-well plate containing 350×10^3 cells/well and 25nM siRNA/transfection reagent complex were added to analyse at a protein level via western blot. Each plate was setup as per figure 2.9. The cell line used for this was the GOhTRT cells, and transfection was carried out as per section 2.3. The seeding densities used were as follows; 5×10^3 cells/well in a 96-well plate, 50×10^3 cells/well for a 12-well plate, and 300×10^3 cells/well for a 6-well plate. Transfection volumes were altered as per Dharmacon protocol. For analysis of complement components cells were seeded in media containing heat inactivated FCS. At 48 hours post-transfection, corresponding wells were serum starved for 3 hours, and then the recombinant protein was added in antibiotic and serum free media. Media was also replaced in all other wells as a control.

Recombinant LIF protein (R&D Systems) was optimised using a range of concentrations indicated in literature. A final concentration of 30ng/mL was found to increase growth significantly as compared to control wells therefore this concentration was used for all further experiments involving recombinant LIF protein.

Native C1q protein (Sigma Aldrich) was optimised using a range of concentrations as indicated in literature. Normal plasma levels of C1q protein in human serum in

~70µg/mL, therefore a range above and below this was used for optimisation purposes. For all further experiments a C1q concentration of 75µg/mL was used.

At 96-hours post-transfection, each plate was assayed individually as described in section 2.14. Briefly, an MTT assay was performed on the 96-well plate. Cells were lysed and an RT-PCR performed on the 12-well plate (as per section 2.9- 2.10). Finally a protein extraction and western blot was performed on the 6-well plate as per sections 2.11 and 2.12. Statistical analysis was performed as per section 2.8, to determine if addition of the recombinant protein increased protein levels as compared to siRNA knockdown wells. Results were normalised to negative control in all experiments.

2.15 Human LIF ELISA for Analysis of Secreted LIF Protein Levels in Cell Supernatant

Cells were grown in a 24-well plate for 96 hours post-transfection with either non-targeting siRNA or LIF siRNA. Supernatant was removed and centrifuged for 5 minutes to remove any remaining particulates. Supernatant was removed and stored at -20°C until needed. Reagents were prepared as per protocol (R&D Systems), and allowed to increase to room temperature before use. To the plate provided, 200µL of each standard, sample and control were added, in duplicate wells. The plate was incubated at room temperature for 2 hours. Each well was washed with wash buffer provided before addition of 200µL of the LIF conjugate. Again the plate was sealed and incubated for 1 hour at room temperature. Each well was washed with wash buffer. Substrate solution was added and incubated for 20 minutes at room

temperature, protected from light. Stop solution was added in which a colour change was noted. The plate was read at 450nm and 570nm. A standard curve was created using data generated from the standard readings, and the amount of LIF protein was then calculated from this for each of the samples used.

2.16 Luciferase Assay

2.16.1 Bacterial Cell Pellet Formation for TOPFLASH/FOPFLASH Luciferase Plasmids

A single colony of bacterial cells containing either; M50 (TOPFLASH) or M51 (FOPFLASH), were chosen from streaked agar plates. A starter culture of LB broth was inoculated with the chosen colony containing either M50 (TOPFLASH) or M51 (FOPFLASH) plasmid and incubated at 37°C overnight on a shaker. The inoculated broth solution was centrifuged at 13,000 x *g* for 15 minutes at 4°C to pellet the cells. This was frozen at -20°C until needed.

2.16.2 Plasmid DNA Extraction

A midi plasmid purification kit (Qiagen) was utilised as per manufacturer's instructions to isolate plasmids for the TOPFLASH/FOPFLASH luciferase assay. Briefly, the bacterial cell pellet containing either the M50 (TOPFLASH) or M51 (FOPFLASH) plasmids was re-suspended in the supplied re-suspension buffer. Lysis buffer was added and the solution was mixed thoroughly until the liquid became viscous. It was then incubated at RT for 5 minutes. Chilled neutralization buffer was added to the lysate and inverted until mixed. This step allows for precipitation of

material including genomic DNA, proteins and cell debris. The solution was immediately poured into the QIAfilter Cartridge and incubated at RT for 10 minutes. In parallel, an equilibration buffer was added to the QIAGEN-tip. A plunger was inserted into the QIAfilter cartridge and the cell lysate was filtered into the equilibrated QIAGEN-tip. The QIAGEN-tip was cleared with wash buffer twice to remove any possible contaminants. The DNA was then eluted with elution buffer which was collected in a 15mL tube which could be stored at 4°C until needed.

2.16.3 Plasmid DNA Precipitation

Isopropanol was added to the eluted DNA for precipitation. The solution was mixed and centrifuged at 13,000 x *g* for 30 minutes at 4°C. Supernatant was removed. The DNA pellet was washed with 70% ethanol, to remove any precipitated salt, and centrifuged at 13,000 x *g* for 10 minutes. The supernatant was removed. The pellet was allowed to air-dry for 10 minutes and the DNA was re-dissolved in 100μL TE buffer. The DNA concentration was calculated using the NanoDrop spectrophotometer.

The M50 (TOPFLASH) plasmid (Fig 2.10A) is a β -catenin reporter with TCF/LEF sites upstream of the luciferase reporter. The vector backbone is a pTA-Luc supplied by Clontech. It is Ampicillin resistant and functions using a luciferase vector. It was generated in the Moon laboratory, along with the mutant plasmid, M51 (FOPFLASH) (Fig. 2.10B). The M51 plasmid is a control for the β -catenin reporter and contains mutated TCF/LEF binding sites upstream of the luciferase reporter. It contains a pGL3 vector backbone supplied by Promega and is again Ampicillin resistant.

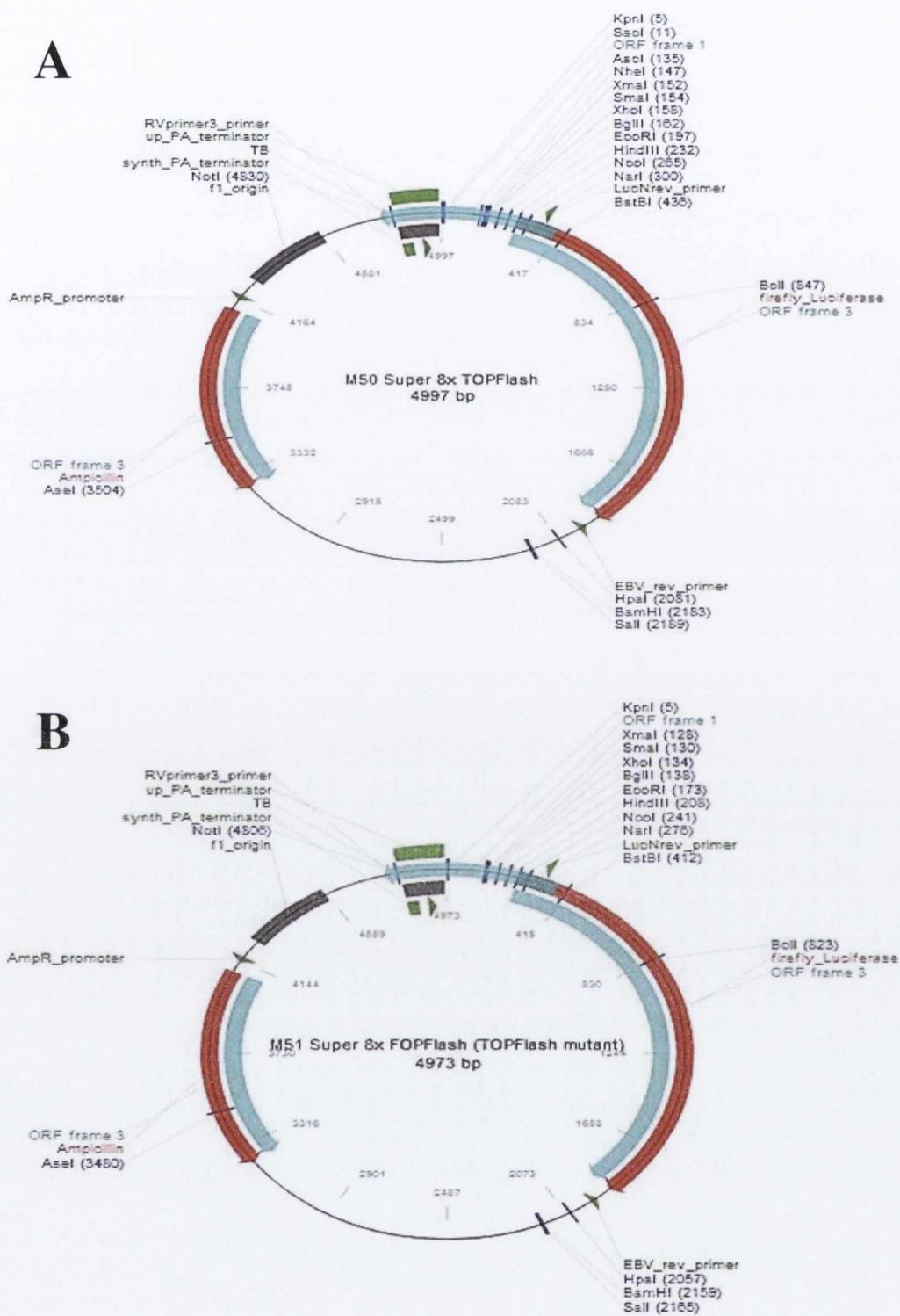


Figure 2.10: TOPFLASH and FOPFLASH Plasmid Map. Luciferase reporter plasmids used for detection of Wnt signalling by luciferase activity. A) M50 TOPFLASH β -catenin reporter plasmid with pTA-Luc vector backbone; B) M51 FOPFLASH control luciferase reporter plasmid with pGL3 vector backbone.

2.16.4 Transfection of Plasmid DNA into Human Embryonic Kidney 293 Transformed Cell Line

FuGene HD transfection reagent (Promega) was utilised and all parameters were optimized previously as per manufacturer's protocol. Briefly, 5×10^3 Hek293T cells were transfected with either the non-targeting siRNA or C1QA siRNA, in a 96-well plate, using 0.2 μ L DharmaFECT 4 for a final siRNA concentration of 25nM. Cells were incubated for 48hrs at 37°C. At 48hrs post-transfection, 0.6 μ L FuGene HD was added to the required amount of blank media, which was then incubated for 5mins. To this, 200ng of the corresponding plasmid DNA was added (M50 or M51) was added. This resulted in a 3:1 ratio of reagent: DNA. The DNA/transfection reagent was allowed to complex for 15 minutes at RT. After the incubation period, 10 μ L of this DNA/transfection reagent mixture was added to each corresponding well of the 96-well plate, and incubated at 37°C for 24 hours.

2.16.5 Dual- Luciferase Reporter Assay

For detection of Wnt signalling following siRNA media knockdown of C1QA, media was removed from the wells and 20 μ L 1x PBL lysis buffer was added to each well. The plate was gently rocked for 15 minutes at RT. The lysate was transferred to eppendorfs for storage at -80°C until needed. For each sample, 20 μ L of the lysate was added to a corresponding well in an opaque white 96 well plate. To this 100 μ L of Luciferase Assay Reagent (LAR) II (Promega) was added. Firefly luciferase activity was read on a luminometer. Change in Wnt signalling following siRNA mediated knockdown of C1QA in the HEK293T cell line was analysed using the luminescence readings generated.

Chapter 3

Results

Optimization of Parameters Necessary

for a Successful Large Scale siRNA

Library Screen

3 Optimization of Parameters for Screen Setup

3.1 Introduction

RNA interference (RNAi) was first discovered in *C. elegans*, in which it was noted that gene silencing could occur due to the presence of double stranded RNA (dsRNA) (Fire et al., 1998b). Following from this discovery small interfering RNAs (siRNA) were shown to guide sequence-dependent endonucleolytic cleavage of mRNA that can regulate mammalian cellular processes (Gavrilov and Saltzman, 2012). Gene interference occurs when long dsRNA is cleaved by R2D2/Dicer heterodimer to form siRNAs. This is then unwound, and the guide strand is taken up by the RISC complex. The single siRNA strand then acts to guide the endonuclease activity of the activated RISC complex to the complimentary site on the mRNA, resulting in mRNA cleavage, and subsequent degradation of the passenger strand.

The development of RNAi technology has resulted in a greater understanding of specific gene functions. This has been highlighted using RNAi mediated gene silencing techniques, using either siRNA and/or shRNA approaches. Short hairpin RNAs (shRNA) integrate DNA and consist of two complementary 19–22bp RNA sequences which are linked by a short loop of 4–11nt which is comparable to the hairpin found in naturally occurring miRNA. Following transcription, the shRNA sequence is transferred to the cytosol where it can be recognized by Dicer, which acts to process the shRNA into the siRNA duplexes (Moore et al., 2010). One of the advantages in using shRNAs is that they can be used to generate stable knockdown cell lines, thus eliminating the need for repeated transfection and increases the reproducibility of results. However, the creation of a stable shRNA cell line is very

time-consuming as the construct preparation and the selection of shRNA-positive cells by drug resistance or fluorescent markers can take a long time. Another disadvantage is that they may cause cellular cytotoxicity through interference of endogenous miRNAs, which has been demonstrated in a mouse model. This study, using a firefly luciferase plasmid, assessed liver-directed shRNA expression and it was noted that although transfection efficiency was high, there was increased morbidity, with some mice dying within a month (Grimm et al., 2006). In comparison, siRNAs can be chemically synthesised or produced *in vivo* or *in vitro*, allowing the siRNA duplex to be delivered directly to the cytosol. Another advantage of siRNA usage is that there are a multitude of reagents available for both siRNA design and synthesis which can yield a potentially high level of gene silencing with low levels of cellular toxicity, allowing for transient transfection (Moore et al., 2010, Wang et al., 2010a). Although siRNA and shRNA both utilise a similar cellular mechanism (RISC), the choice of which method to use depends on a number of factors including cell type, time constraints, and the need for either transient or stable integration. Given that we did not require a stable transfection to assess effect on OAC cell viability, that time demands were tight, and that we wanted minimal cellular toxicity, the use of a siRNA library was chosen, over a shRNA library. Also, siRNAs were successfully utilised for gene targeting within OAC cells lines previously within the lab.

The use of RNAi technology has thus been harnessed and siRNAs are now widely commercially available from various companies. Libraries of siRNAs can be arrayed in 96, 384 or 1536-well format and can be constructed to cover particular functional sets of genes. For example, sub-libraries within the Dharmacon genome library used

by us include the human druggable genome, kinase family members and finally the G-protein coupled receptors (GPCR) library. The druggable genome library contains a selection of siRNAs targeting 6,022 genes that are known to express proteins which can bind drug-like molecules (druggable) (Hopkins and Groom, 2002). Although these targets are defined as '*druggable*', some may not be actual drug targets; unless a gene is also linked to a disease it is not termed as a '*drug target*'. This thesis aimed to identify novel therapeutic targets for the treatment of OAC and therefore we felt that the use of the druggable genome would facilitate this. Library-based screening studies can utilise a variety of end-points including cell viability, effects on cellular morphology or protein localisation. For example, a whole genome library was used to identify nucleosporin 62 as an inhibitor of viral morphogenesis as determined by immunofluorescence staining (Sivan et al., 2013), and a druggable genome library was also used to identify four genes which modulate cell proliferation in epithelial ovarian cancer, as determined by CellTiter Blue assay (Sethi et al., 2012).

Each step of the transfection process requires optimization including; determination of cell type and number, along with identification and optimisation of transfection reagents, and transfection conditions. Previous work within our laboratory had utilised a lipid based siRNA protocol with DharmaFECT siRNA transfection reagents to transfect the siRNA into the cell (lipofection). This method utilises cationic lipid molecules that form unilamellar liposomes which negatively interact with the nucleic acids (Felgner et al., 1987). They contain hydrophobic and hydrophilic regions which allow for the formation of spheroid liposomes under aqueous conditions (Zhou, 2013). The lipid based transfection reagent can then be taken-up by the cell by

endocytosis and subsequently released into the cytoplasm (Mortimer et al., 1999, Yadava et al., 2008).

There are a number of other transfection methods available. For example, electroporation uses a voltage pulse to target the plasma membrane allowing access to the cytoplasm, while Nucleofection uses the same principle but utilises a series of pulses which allows access to the nucleus (Washbourne and McAllister, 2002). Virus based transfection methods utilising adenoviruses or lentiviruses, generally result in high transfection efficiency but they require biosafety level 2 facilities and a lot of recombinant vector preparation is necessary which is extremely time consuming (Karra and Dahm, 2010). Physical transfection methods can also be utilised such as microinjection and biolistics. Both methods inject the cell with the RNAi, but microinjection uses a fine glass capillary to facilitate this in individual cells (Zhang and Yu, 2008), while biolistics uses DNA-coated gold particles using a motive force (Lo et al., 1994). Given this information, lipofection was chosen as the transfection method of choice for several reasons; it is cost-effective, it is a simple and fast procedure, it is suitable for stable and transient transfection, and it shows high reproducibility rates with low toxicity levels.

Off-target effects (OTEs) are defined as the unwanted down-regulation of non-target mRNA. Although the risk of OTEs is minimal in commercially available pooled siRNAs, precautions are still a necessity. It has been shown that reducing siRNA concentration can help avoid OTEs such as toxicity that is not due to transfection. It has been identified that pooling of multiple siRNA sequences, each targeting the same gene, shows strong on-target gene knockdown while reducing the concentration of

siRNA needed, thus incurring minimal OTEs. The success of pooling in RNAi experiments was shown by the development of esiRNAs. Endoribonuclease-prepared siRNAs (esiRNA) are a mixture of siRNA oligos with an endoribonuclease (RNase III or dicer) derived from *E. coli*. When testing the esiRNAs, it was noted that pooling of siRNAs would achieve the same level of specificity as the esiRNAs (Kittler et al., 2007). Hence many commercial synthetic siRNAs, such as those in the Dharmacon SMARTpool library, which we have utilised for our studies, contain pooled siRNA sequences.

Each cell line is different; therefore efficiency of siRNA uptake differs. One of the variables is the sensitivity of different cell lines to genetic modifications (McManus and Sharp, 2002). The main aim of siRNA transfection is to maximise silencing and therefore optimization of conditions favouring this outcome are essential. These optimization experiments include identification of conditions favouring good sensitivity, low variability and significant signal-to-noise separation (Echeverri and Perrimon, 2006). The dynamic range is then calculated and a screen is deemed successful if the separation between known negative and positive controls is statistically significant. Efficient controls are highly important for successful gene silencing. A negative control reference should include cells transfected with a non-targeting or inactive siRNA. A non-targeting negative control contains the same nucleotides as active siRNA, but they are not in a logical order; they are '*scrambled*' and therefore the resulting sequence is not complementary to any known RNA transcript (Grinev, 2012).

Positive controls are used to determine quality of the transfection. Such controls play a key role in selection-based screens and should be included in all plates in a screen to monitor data quality. The inclusion of positive controls allows for determination of screen quality to ensure that all plates and wells are subject to equal conditions and stringency (Echeverri and Perrimon, 2006). Previous work within our laboratory by Dr. Fiona Behan identified several genes that could act as possible positive controls for use in the GOhTRT high grade dysplastic cell line. GATA6 and Bcl-xL showed statistically significant reduction in cell viability assessed via MTT assay; therefore these genes were carried forward for assessment within optimization experiments before the screen could be setup. GATA6 is a transcription factor which plays a role in cellular differentiation during embryonic development. Amplification of this gene has been shown in OAC, while genetic silencing using an siRNA for GATA6 results in significant reduction in cell proliferation (Lin et al., 2012). Bcl-xL is a member of the Bcl-2 family which act as either pro- or anti-apoptotic regulators. Increased expression of Bcl-xL has been shown in IHC studies of BO tissues, with increasing levels shown in the sequence from dysplastic to adenocarcinoma (Beales and Ogunwobi, 2009). For this body of work, a successful positive control would reduce cell viability significantly, but would not completely kill off the cells, thus allowing for quantifiable indications of reduction in cell viability. Reproducibility within the both the negative and positive control is also of high importance as it indicates baseline values from which to compare samples results. The use of positive controls confirms the transfection efficiency for each transfection (Ovcharenko et al., 2005). Successful implementation of this is shown in a wide range of siRNA screen optimisations. One study used PLK1 as a positive control for its effectiveness to kill HeLa cells (Stec et al., 2012), while another used UBB due to its universally lethal

effect on cell lines (Zhu et al., 2011). The latter study also utilised the human druggable genome siRNA library. The screen optimisation steps undertaken within this body of work are similar to literature published from other library screens. All screens need to optimise parameters using pilot studies to titrate assay variables for assay response to negative and positive control siRNA molecules. This is a standard pre-requisite step necessary for confident progression to hit selection and validation.

Statistical analysis of the raw data generated is necessary to determine the quality of the screen (*quality control/QC*), by analysing the performance of the controls. Several tests can be utilised; normalisation, t-test, Z-factor and SSMD. Data must be normalised to correct for any differences that may be caused due to day-to-day variation. There are several types of normalisation, for example normalisation to the median of the negative control is optimal for analysis of effect on cell viability, used throughout this thesis. This method demonstrates consistently similar effect size, and is the best strategy of adjustment of spatial effects. It was necessary to use statistical analyses that would take into account data variability (standard deviation). The first statistical test, Z-factor, is widely used in RNAi analysis for analysing data quality. It determines the dynamic range which is the difference between the means of both the negative and positive controls. Analysis of the dynamic range allows for assessment of how well the assay performed. The second test, SSMD, uses the standard deviation of the difference between both the negative and positive controls. This differs from the Z-Factor which uses the sum of the standard deviation of both controls. The third test used, t-statistic, assumes that data has a central t-distribution with 'n-1' degrees of freedom, and a null hypothesis of zero mean difference. It determines mean difference

between both positive and negative controls, hence analysing the significance of the reduction in cell viability.

3.2 Hypothesis and Objectives

Sufficient optimisation of all transfection and statistical parameters is necessary before a screen can be implemented to reduce the risk of OTEs resulting in the identification of high confidence hits. This leads to the question, what are the optimum parameters necessary for the successful upscaling of the siRNA transfection from a single 96-well plate to 228 96-well plates? Thus this chapter aims to determine the optimal conditions under which to perform a siRNA screen of the human druggable genome, by extensive analysis of the cell lines available, methods for delivery of siRNAs, and ways in which to reduce time on the setup day. These aims were achieved through:

- Optimization of growth and transfection conditions for GOhTRT cell line
- Development of methods to improve efficiency of the screen
- Identification of effective positive and negative controls
- Determination of the most effective plate layout for the screen setup to reduce systematic errors
- Basic quality control analysis with the potential to be up-scaled for a larger screen
- Overall determination of the most efficient process under which to perform the screen itself

3.3 Results

Optimal siRNA transfection conditions vary between cell lines and therefore identification of these optimal conditions must be identified for each cell line used throughout this study. The experiments necessary for identification of these conditions, outlined in Figure 3.1, include; optimization of cell seeding density, transfection reagent, siRNA concentrations and stability, while determination of efficacy of positive and negative controls was also necessary. Pilot screens allow for identification of optimal plate layout and reproducibility of controls when setup in batches. Establishing suitable statistical tests for the rigorous analysis of data output was also necessary.

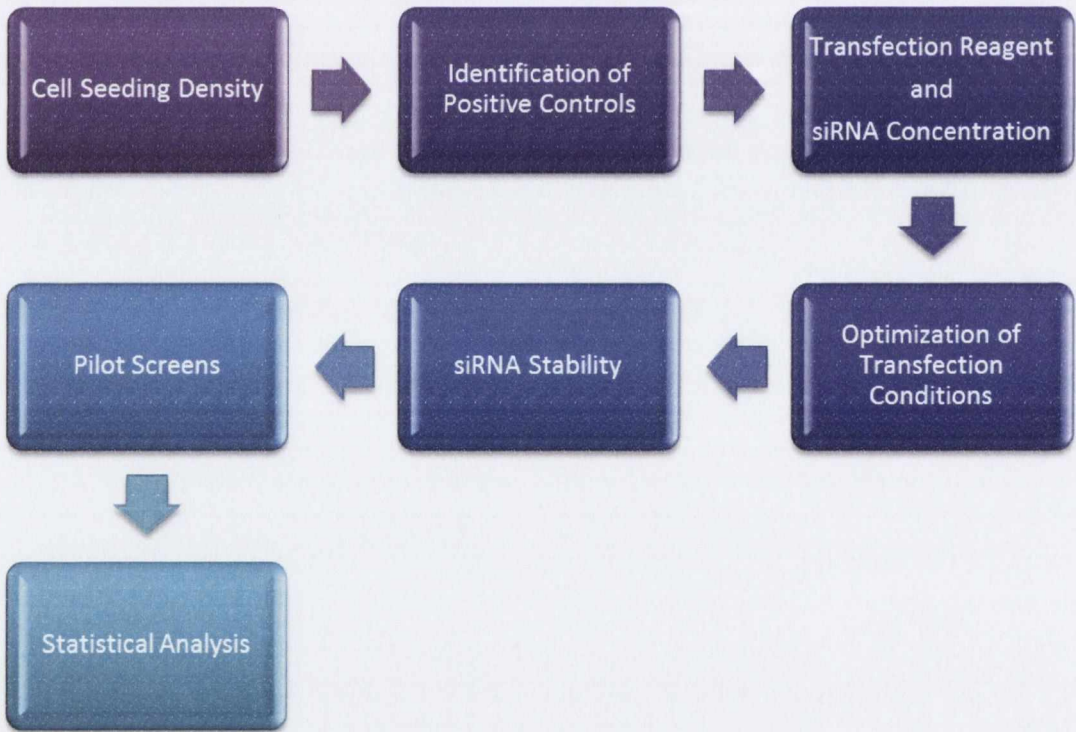


Figure 3.1: Experiments Necessary for Development of Screen Platform. Several steps necessary to determine optimal screen conditions.

3.3.1 Identification of Optimal siRNA Transfection Conditions in GOhTRT Cell Line

A seeding density of GOhTRT cells that would allow for continued cell proliferation up to 96 hours had to be determined. The GOhTRT cell line was chosen, as previous research by Dr. Fiona Behan in our lab had observed that this cell line showed resistance to a DCA induced reduction in cell proliferation. A range of cell densities of GOhTRT were chosen, plated in 96-well plates and incubated (untreated) at 37°C with 5% CO₂ for 24-96 hours (Figure 3.2). Highlighted data shows the optimal seeding density chosen, of 5x10³ cells per well, incubated for 96 hours, which was used for all subsequent experiments. This seeding density allowed for continued reproducible cell growth up to 96 hours.

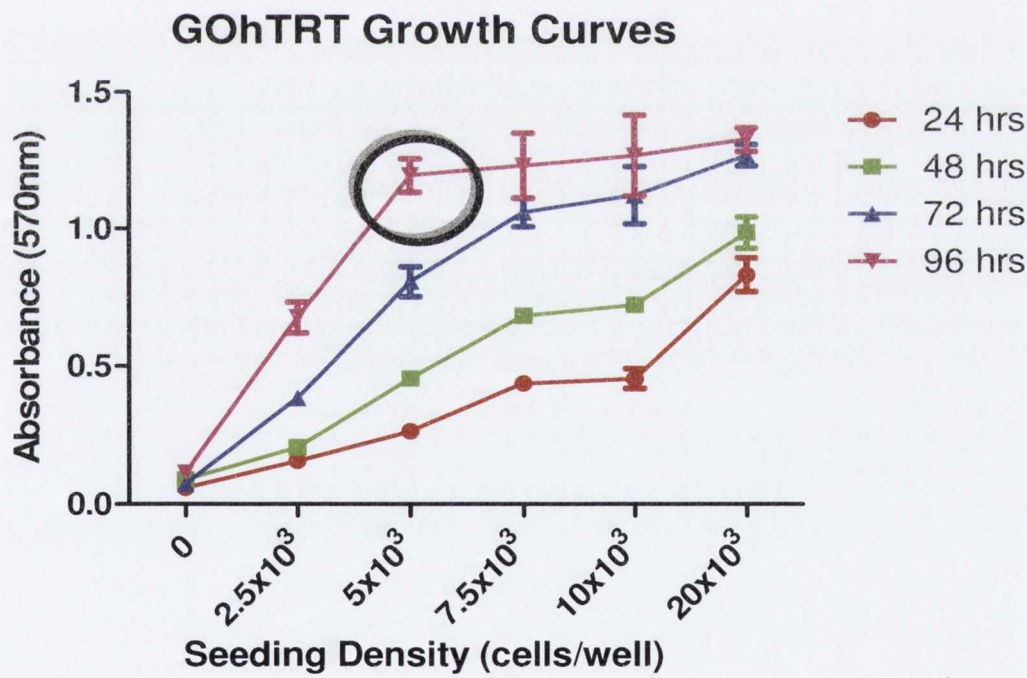


Figure 3.2: Seeding Densities. Cells seeded at varying densities, and incubated for a range of periods, 24-96 hours. N=3, in triplicate wells ±SEM. Cell viability was determined via MTT assay.

Four lipid based transfection reagents (DharmaFECT 1-4) were available for use with Dharmacon reagents and thus identification of the transfection reagent that resulted in optimal transfection efficiency was necessary. The manufacturer's instructions indicate the use of 25nM, therefore a range of siRNA concentrations were analysed ranging from 100nM to 25nM. This experiment was setup in GOhTRT cells, with a seeding density of 5×10^3 cells per well, as previously optimized (Figure 3.2). The DharmaFECT transfection reagent was used as recommended at a volume of 0.2 μ L in 9.8 μ L of media without supplements. Each siRNA was then diluted to the desired final concentration in media without supplements. The siRNA and transfection reagents were then allowed to complex and delivered to cells as described in section 2.3. Plates were incubated for 96 hours at 37°C and 5% CO₂. Results are shown in Figure 3.3, with optimal transfection noted at siRNA concentration of 25nM using DharmaFECT4 (highlighted with red circle) indicating a reduction in cell viability of 46% for GATA6 ($p < 0.05$) and 73% for siTOX ($p < 0.05$). Thus, in all further experiments, a siRNA concentration of 25nM and a DharmaFECT 4 volume of 0.2 μ L were used in the GOhTRT cell line. A selection of efficient positive and negative controls is also necessary for a successful assay. Results showed that cell viability was significantly reduced by inhibition of both GATA6 (66%) and Bcl-xL (58%), as compared to the non-targeting negative control (Figure 3.4). Therefore both were deemed as good quality controls for use with the GOhTRT cell line, and used as such in all further experiments.

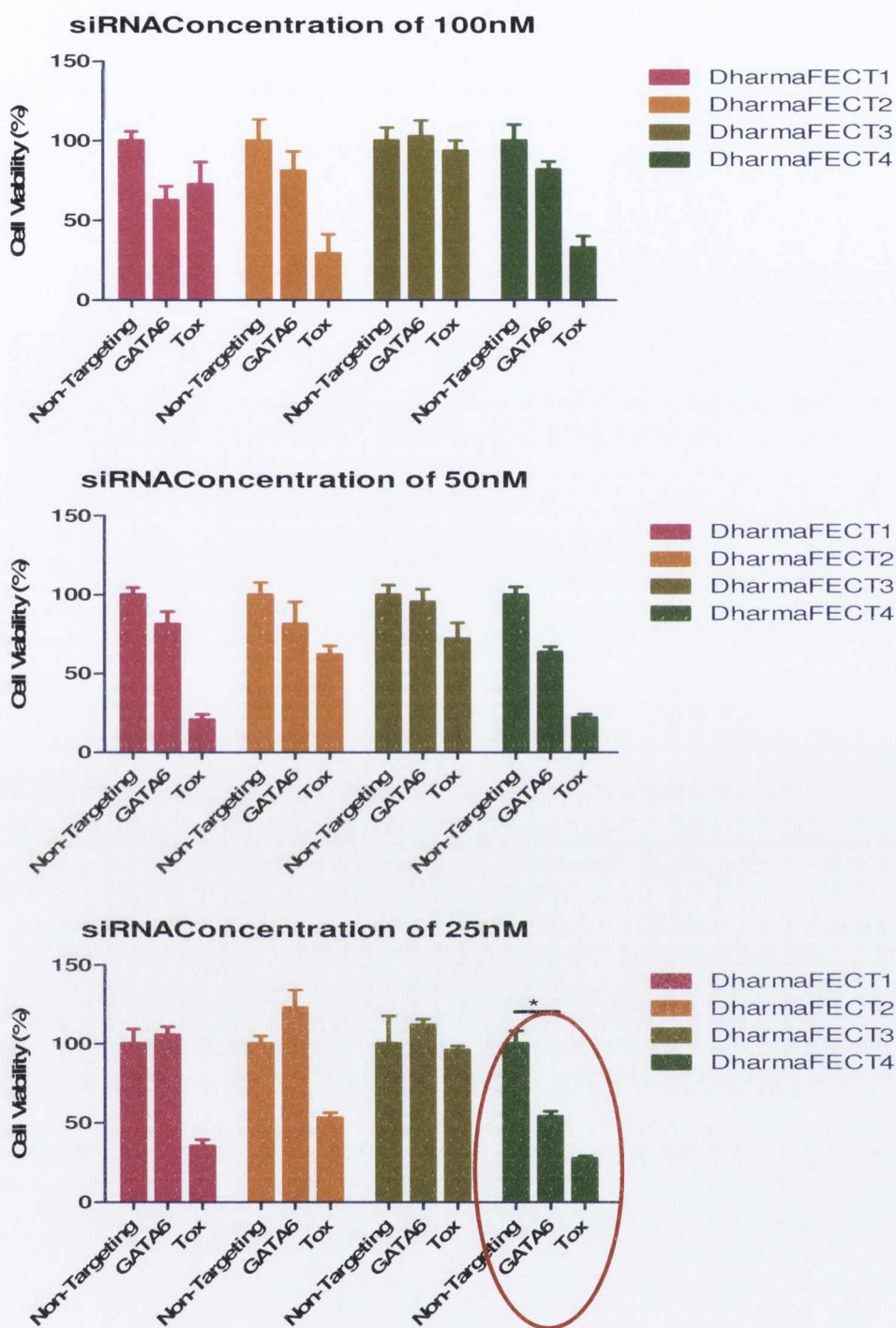


Figure 3.3: Transfection Reagent Optimisation. GOhTRT cells were seeded at 5000 cells/well, and siRNA and transfection reagent were diluted to desired final concentration using media without supplements as described in section 2.3. Cell viability was assessed by MTT assay and analysed using the t-test. N=3 in triplicate wells $\pm$ SEM. Negative control: Non-Targeting; Positive controls: GATA6 and siTOX. * $p < 0.05$

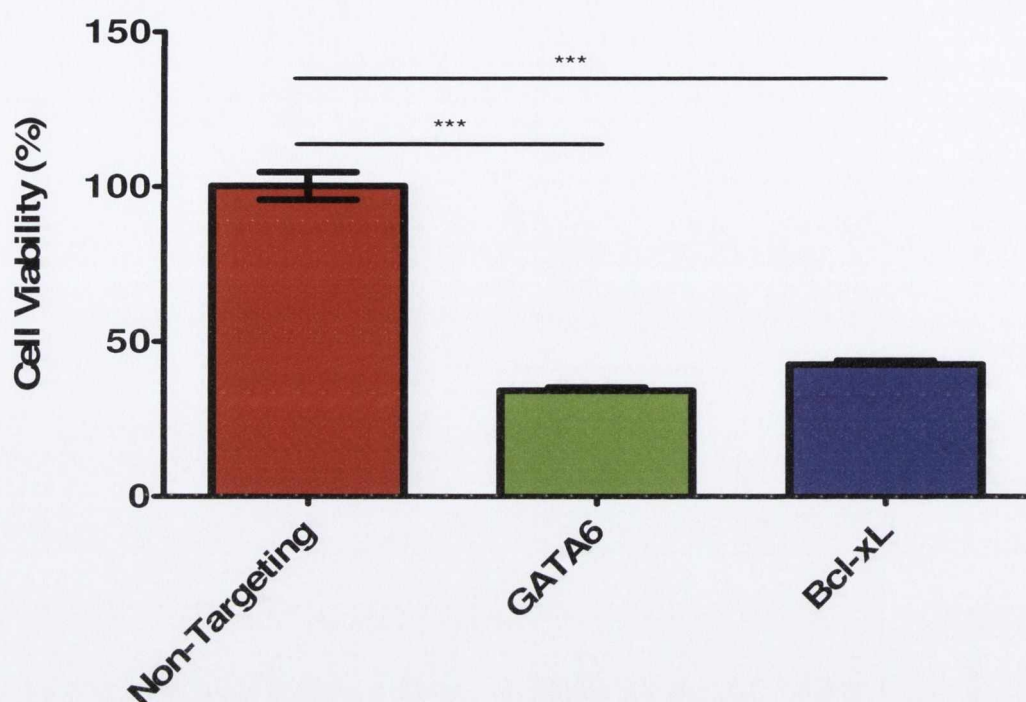
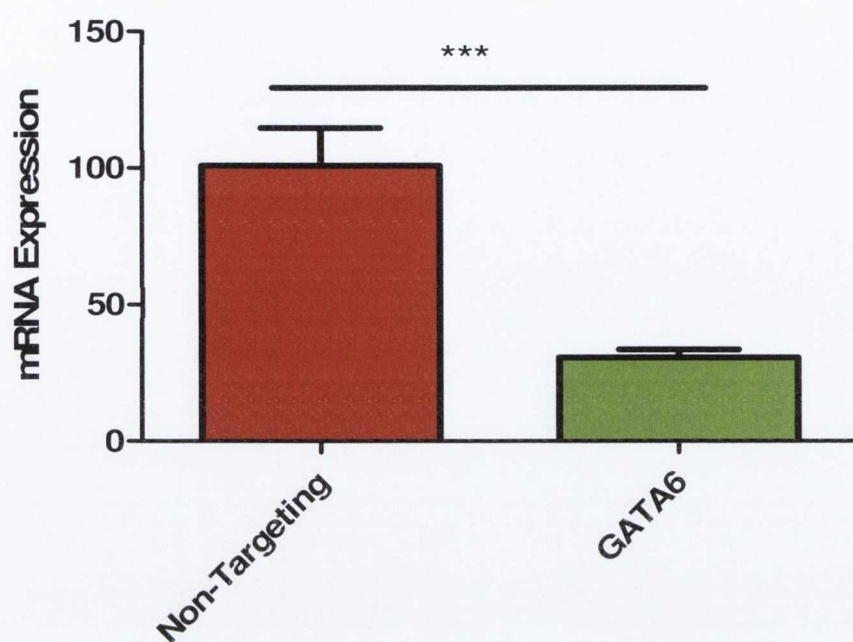


Figure 3.4: Confirmation of Efficacy of Positive Controls on GOhTRT Cell Line. Non-Targeting, GATA6 and Bcl-xL siRNAs were used at a concentration of 25nM, and incubated for 96 hours at 37°C as per section 2.3. Seeding density was 5×10^3 cells/well. Cell viability was quantitated using the MTT assay and analysed using the t-test. N=3, with 3 replicate wells $\pm$ SEM. (***)P < 0.0001).

Confirmation of the knockdown of these controls at both an mRNA and protein level was also undertaken using RT-PCR (Figure 3.5) and Western blot (Figure 3.6), respectively. At an mRNA level significant knockdown was achieved for both genes, GATA6 (70%) and Bcl-xL (53%). Cells were incubated for 48 hours post-transfection before RNA extraction as per sections 2.9 & 2.10. Western blot experiments were utilised to detect protein knockdown. Densitometry was performed for both western blots for a graphical interpretation of the level of protein knockdown (Figure 3.6).

A



B

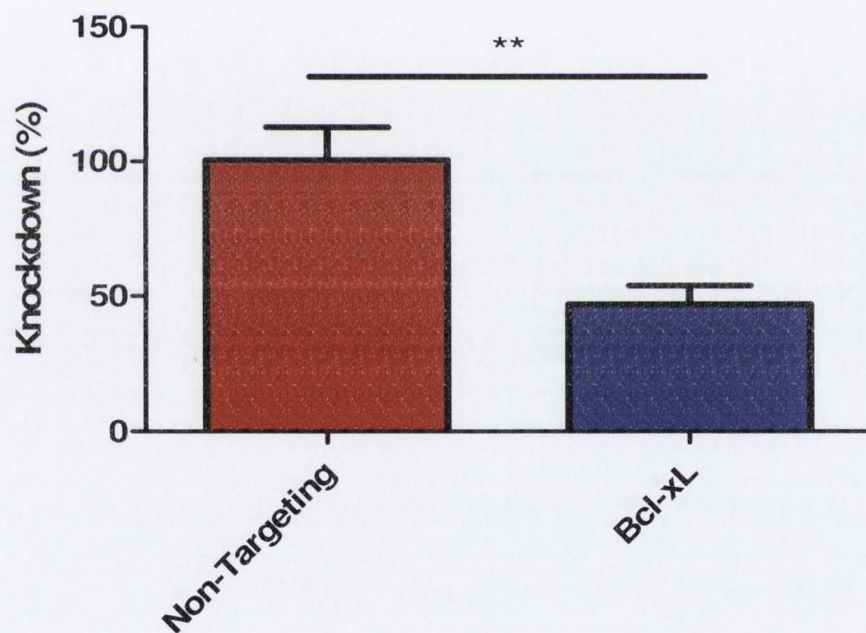
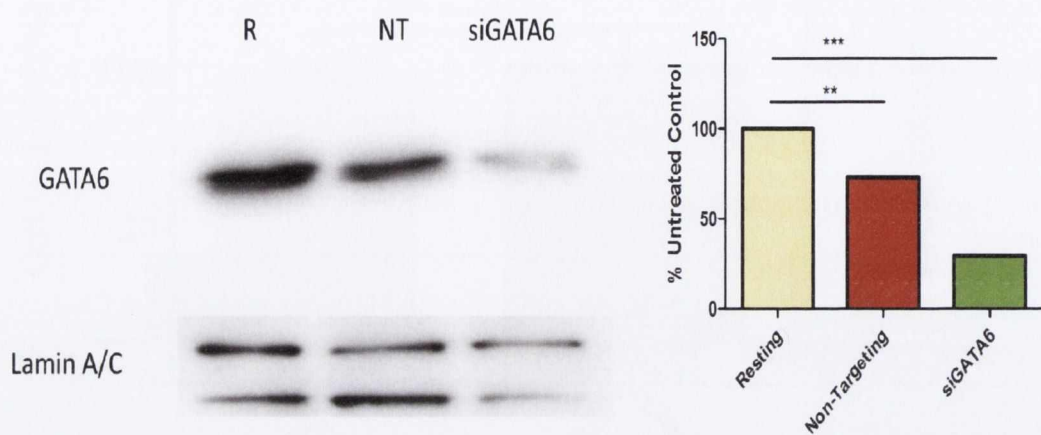


Figure 3.5: Confirmation of GATA6 and Bcl-xL Knockdown at mRNA Level. A) Confirmation of the knockdown of GATA6 by 70% as compared to the non-targeting negative control was demonstrated by RT-PCR, while B) determination of Bcl-xL knockdown of (53%) was determined in the same way. Non-targeting siRNA was used as a negative control. N=3 $\pm$ SEM, analysed using t-test (**P=0.0027, ***P < 0.0001)

A



B

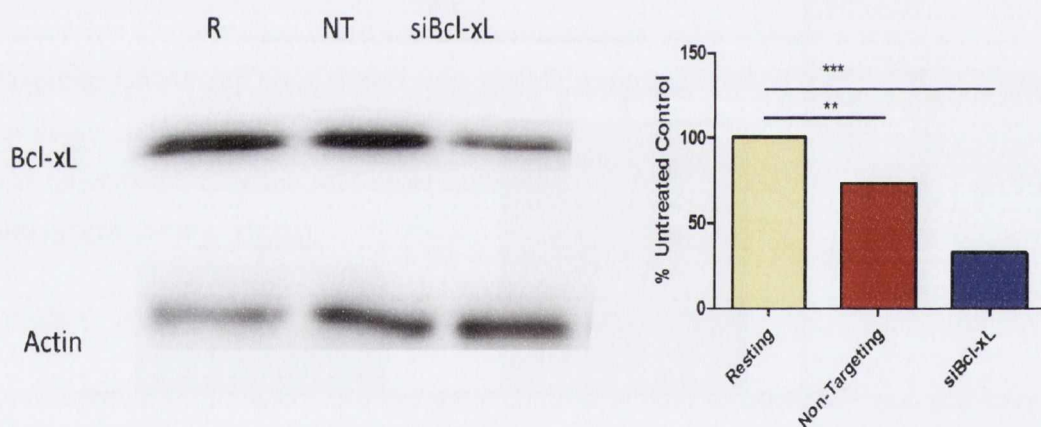


Figure 3.6: Confirmation of GATA6 and Bcl-xL Protein Knockdown. Confirmation of protein knockdown in the GOhTERT cell line. A) GATA6 within the nuclear fraction and B) Bcl-xL within the cytoplasmic fraction. HepG2 cells were used as positive controls in both experiments as the cell line expresses both GATA6 and Bcl-xL highly. β -Actin serves as a loading control for cytoplasmic fraction. Lamin A/C serves as loading control for nuclear fraction. Densitometry was performed and is shown as percentage of resting cells. $N=3 \pm$ SEM, and analysed using t-test. (** $p < 0.01$, *** $p < 0.001$).

3.3.2 Determination of Transfection Setup, Storage and Incubation

Conditions of siRNA Transfection Plates

The Dharmacon siRNA manufacturer's protocol specified a 20 minute incubation period for the transfection reagent with the siRNA and cell exposure at 37°C for 72 hours. Previous work in the lab had shown that a phenotypic change occurs at 96 hours post-transfection (Figure 3.7) when using GATA6 as a positive control in the GOhTRT cell line. Cells appear elongated compared to the negative control rather than exhibiting apoptotic rounding. Therefore it was necessary to firstly analyse whether the length of time allowed for the siRNA and transfection reagent to complex affected the efficacy of the transfection. In conjunction with this, the overall time length of incubation of the transfected cells at 37°C was tested to see if it altered the efficacy of the transfection on cell viability. The GOhTRT cells were seeded at 5×10^3 cells/well, while a siRNA concentration of 25nM, using a DharmaFECT 4 volume of 0.2µL were delivered to the cells as indicated in section 2.3. Four plates were setup in parallel to test each parameter;

- 1) 20 minutes for complex of siRNA/transfection reagent then incubated for 72 hours;
- 2) 30 minutes for complex of siRNA/transfection reagent then incubated for 72 hours;
- 3) 20 minutes for complex of siRNA/transfection reagent then incubated for 96 hours;
- 4) 30 minutes for complex of siRNA/transfection reagent then incubated for 96 hours.

Following the different incubation periods (72 or 96 hours), an MTT assay was performed to determine effect on cell viability. As highlighted with the red circles (Figure 3.8), it was determined that 20 minutes incubation of the siRNA and transfection reagent, along with incubating the plates for 96 hours post-transfection

resulted in the most pronounced reduction in cell viability using our positive control, GATA6 ($p < 0.05$). The results indicated a reduction of $47\% \pm 0.95$ for GATA6 and a reduction of $38\% \pm 0.26$ for Bcl-xL in cell viability as compared to the non-targeting siRNA treated cells. Therefore all subsequent siRNA transfections using the MTT assay followed these time periods.

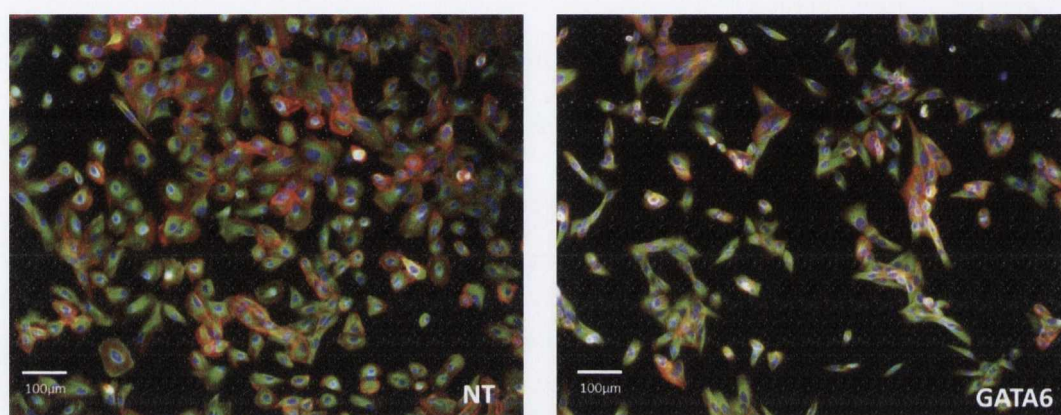


Figure 3.7: Phenotypic Change After siRNA GATA6 Knockdown in GOhTRT Cells. A phenotypic change is noticeable between the non-targeting treated cells (left) and GATA6 silencing (right). Cells appear more elongated when GATA6 is silenced via siRNA. Actin: Red; Tubulin: Green; Hoechst: Blue. Images were taken at 10x magnification using the InCell analyser 1000.

The next step was to analyse the stability of the siRNAs when frozen at -20°C in V-bottom polypropylene plates, at a concentration of $5\mu\text{M}$. It has been demonstrated that lipoplexes are unstable in liquid suspension for long periods of time, therefore necessitating their preparation immediately prior to use (Anchordoquy et al., 1997). For long-term storage, siRNAs are usually kept in a lyophilized state which can be easily re-suspended to a specific concentration. The siRNA is then aliquoted in small volumes to reduce the number of freeze/thaw cycles. Manufacturer's instructions suggest that they should not be freeze/thawed more than 15 times, but for optimal

performance recommends no more than 5 freeze/thaw cycles (ThermoScientific, 2008). By setting the siRNA concentration to 5 μ M before the screen, this allows for a simple dilution to 25nM by addition of media and transfection reagent. This concentration of 25nM was previously identified as optimal in Figure 3.3. Non-targeting and TOX siRNAs were used to test the stability of the siRNAs frozen at 5 μ M in a V-bottom polypropylene 96-well plate. After storage at -20°C for 3 days, the plate was thawed and transfection was setup as per section 2.3, using non-targeting and TOX siRNAs at 25nM, in GOhTRT cells. An unfrozen plate, using fresh non-targeting and TOX siRNAs was setup on the day of transfection, as per section 2.3. Both plates were setup in parallel and the 'unfrozen' plate was used as a control. Results showed (Figure 3.8) that freezing the siRNAs for 3 days, at -20°C did not significantly affect transfection efficiency as compared to when the siRNA is prepared fresh on the day of transfection. Therefore, to reduce labour on the day of the screen, the siRNA library would be re-suspended to 20 μ M concentration, aliquoted into polypropylene V-bottom plates at a pre-calculated volume of 1 μ L in 3 μ L 1x siBuffer, heat sealed, and frozen at a final concentration of 5 μ M. Plates would then be thawed for use on day of the screen.

Reverse transfection protocols recommend adding cells to the siRNA complex which is already incubating for 20 minutes in the wells. If the cells could be seeded before adding in the siRNA complex, with minimal change to the efficacy of the transfection, time management would be better due to the strict incubation periods for the complex. Therefore we aimed to determine whether the order of addition affected transfection efficiency. Results showed (Figure 3.10) that there was no significant difference between each parameter tested. Therefore, to reduce time on the day of the

screen cells would be added to the transfection plates first. The siRNA/transfection reagent complex would be added to the pre-plated cells.

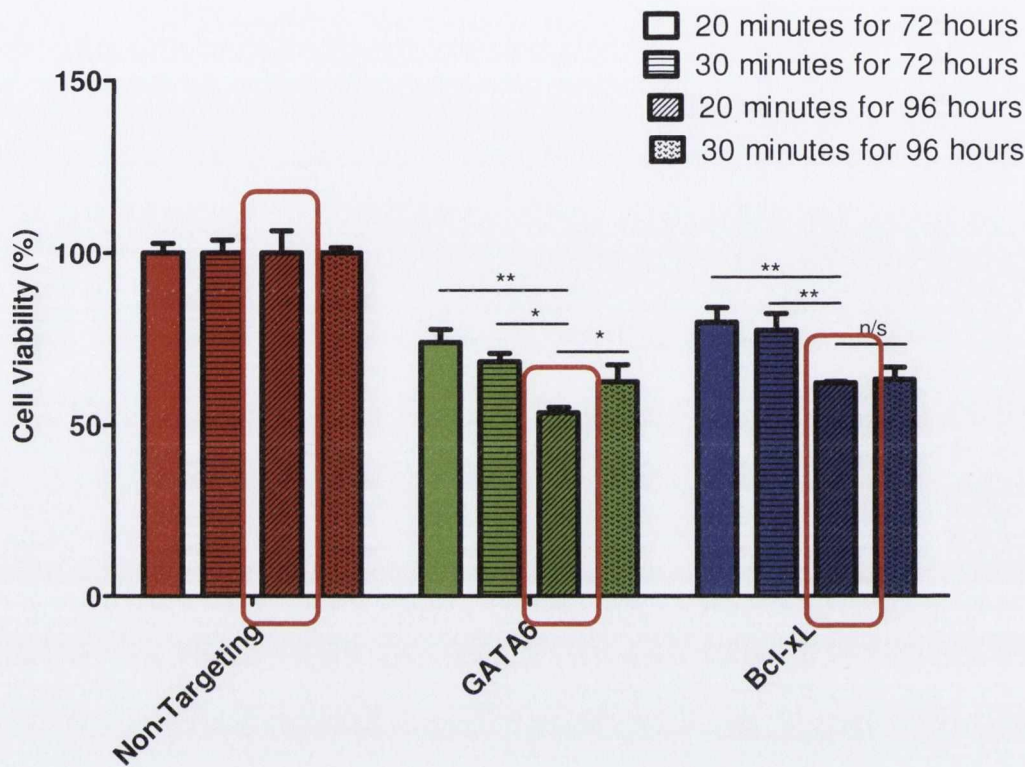


Figure 3.8: Determination of Optimal Incubation Periods ; For both complex of the siRNA and transfection reagent, along with incubation for transfection efficacy. The siRNAs were allowed to complex with the transfection reagent for either, 20 minutes or 30 minutes, then incubation with the cells for either, 72 hours or 96 hours, at 37°C. Cell viability was assessed by MTT assay. Non-targeting siRNA was used as a negative control. GATA6 and Bcl-xL were used as positive controls. N=3, in triplicate wells $\pm$ SEM, and analysed using t-test.

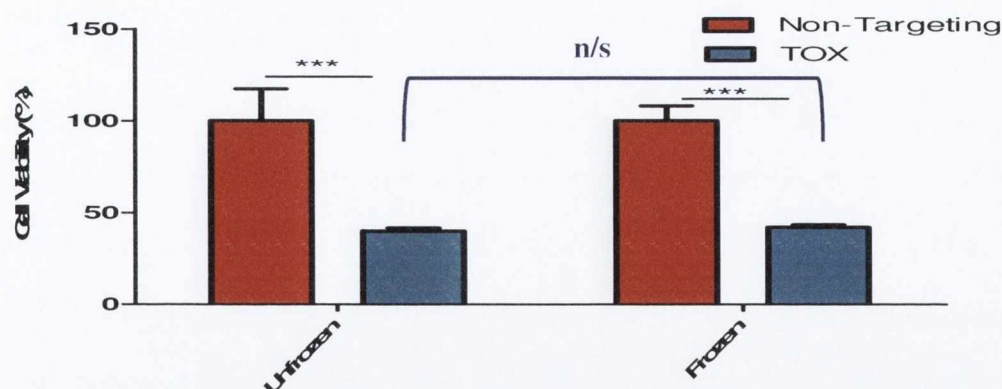


Figure 3.9: Stability of the siRNAs After Freezing over Several Days. ‘Unfrozen’ plate was setup as per section 2.3 on the day of transfection. The ‘frozen’ plate contained non-targeting and TOX siRNAs stored at a concentration of 5µM for 3 days at -20°C. GOhTRT cells at a seeding density of 5x10³ cells/well were added. Cell viability was assessed using the MTT assay. N=3, with three replicate wells were used in each plate and analysed using t-test (P < 0.0001).

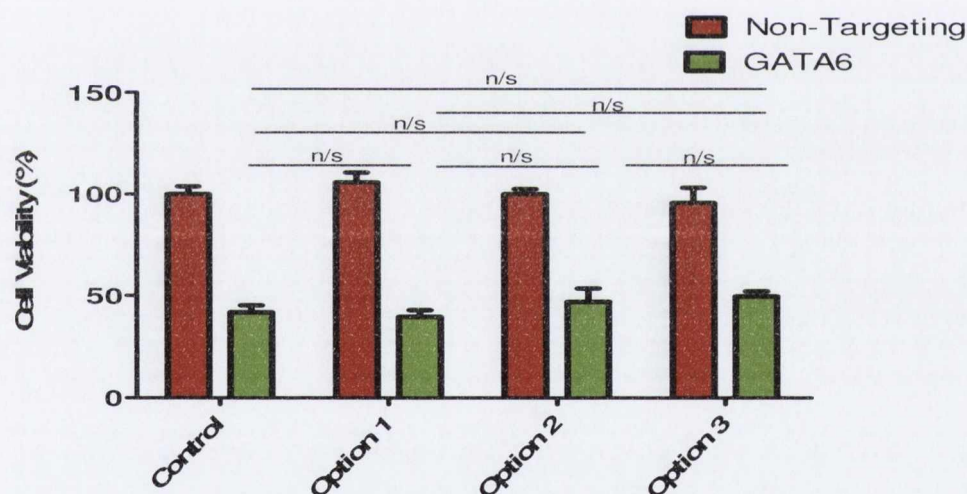


Figure 3.10: Identification of the Best Order of Addition of Both the siRNA Complexes and Cells. Four options were setup to determine if the order of addition of the reagents and cells affected the transfection efficacy. Control: Complex the siRNA and DharmaFECT in an eppendorf, add to wells, then add cells on top; Option 1: Add siRNA to well, then DharmaFECT and allow to complex, then cells on top; Option 2: Add DharmaFECT, the siRNA and allow to complex, then add cells on top; Option 3: Complex siRNA and DharmaFECT in eppendorf, and add to cells there seeded in wells 5 minutes beforehand. N=3, in triplicate wells

3.3.3 Pilot Screens to Analyse Effect of Up-scaling on Optimised Protocol

The siRNA library plates were provided pre-plated with the 6022 lyophilized siRNAs in columns 3 to 12, leaving only column 1 and column 2 empty for addition of controls (Figure 3.11). To reduce the risk of systemic or spatial effects, controls should be randomly dispersed across the plate. This option was not available to us, therefore it had to be determined which column, 1 or 2, would be the best to place to position the controls. For this reason we used pilot screens to determine; which column was best (Figure 3.12B), and also examine reproducibility of results on a larger scale (Figure 3.12A) following statistical analysis.

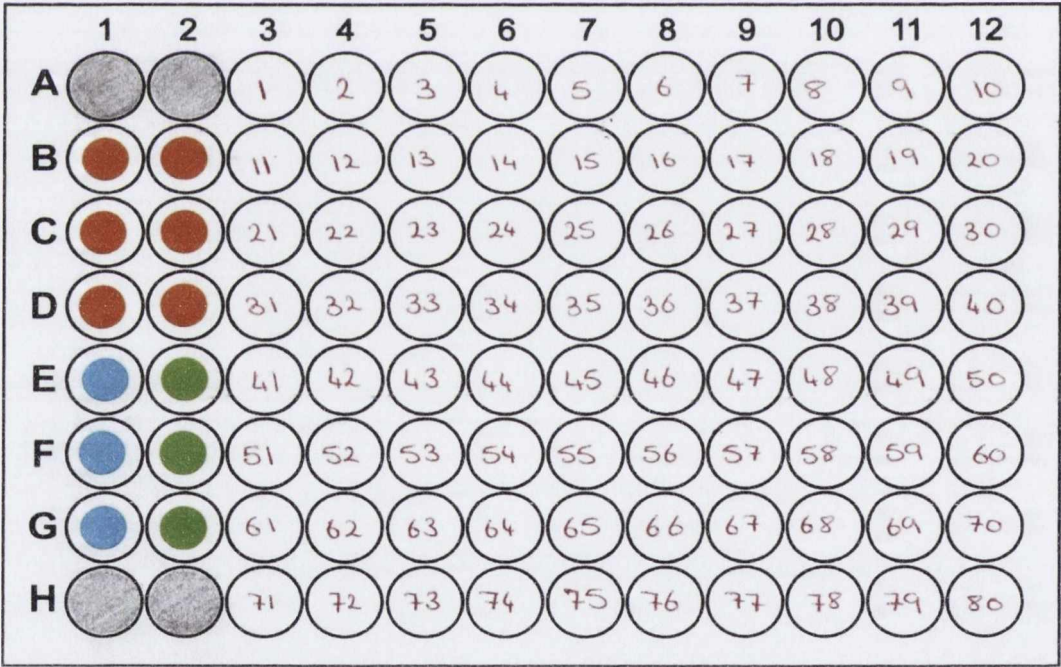


Figure 3.11: Plate layout for Screen. Wells are filled by row as shown above. The druggable genome library has siRNAs filling columns 3-12, leaving columns 1 and 2 available for addition of controls. Orange: Negative Control; Blue: Bcl-xL; Green: GATA6; Grey: Resting Cells

3.3.3.1 Identification of Optimal Plate Layout

It has been reported that to reduce the risk of systematic errors such as plate-to-plate variation or spatial effects (e.g. liquid evaporation in edge wells), the controls should be randomly dispersed across the plate (Zhang, 2011b). To assess this, 4 transfection plates were set up from 1 daughter plate, for a total of 8 transfection plates from 2 daughter plates as per section 2.5. Figure 3.12A shows the reproducibility of the controls when used in a pilot screen, testing only the controls. Overall there is a clear separation between the negative and positive controls, with the majority of the positive control values below 50%, which correlates well with Figure 3.4. This indicates that transfection plates set up at different times or in batches, still retain a constant significant reduction in cell viability.

The next step was to analyse which column available for addition of controls would be best for successful gene inhibition, and to minimise systematic spatial errors. This analysis was carried out using Bcl-xL as a positive control only. It is clear from Figure 3.12B, that there is no significant difference between the positive controls in columns 1 & 2 ($p=0.6039$). Due to the non-significant result obtained, it was concluded that controls placed in column 2 should be used as this would reduce the risk of systemic effects such as evaporation of media liquid from edge wells. This also correlates with a previous report (Zhang, 2011b), suggesting that if only edge wells were available, that the second column should be used instead of the first column.

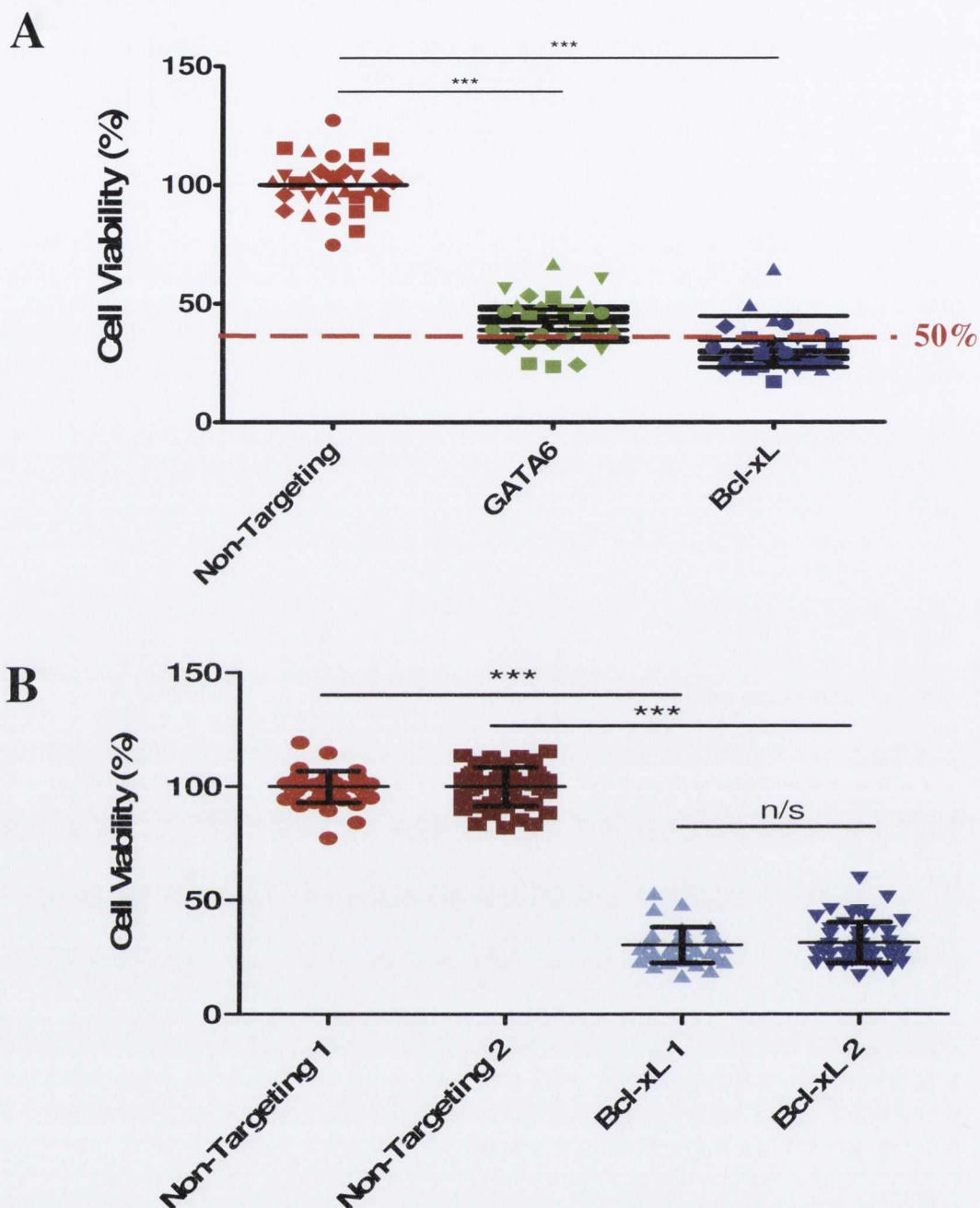


Figure 3.12: Pilot Screen to Analyse Repeatability of Controls and Optimal Plate Layout. Pilot screens were setup to analyse; A) Repeatability and efficiency of controls when setup in batches. A clear separation was noted between the negative and positive controls with the majority of the positive control values below 50% (t-test; $p < 0.0001$); B) Analysis of which column would result in least systemic effects for addition of controls. No significant difference noted between columns 1 & 2 when 5 'library' plates were setup resulting in 20 'transfection' plates (t-test; $p < 0.0001$). $N=3$. Non-targeting siRNA was used as a negative control. GATA6 and Bcl-xL were used as positive controls.

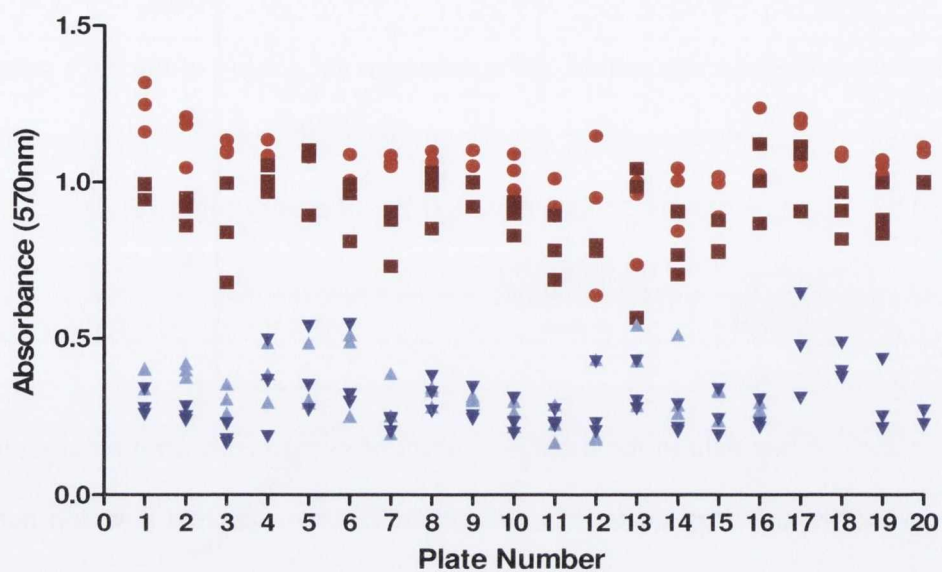
3.3.4 Statistical Analysis of Pilot Screen Data

Basic statistical analysis was carried out to determine the quality of the pilot screens, which included; normalisation of the raw data to the median of the negative controls, followed by utilisation of t-test, Z-factor and SSMD for quality control analysis.

3.3.4.1 Normalisation

Normalisation of raw data to the negative control takes into consideration any plate-to-plate variation that may occur. The screen itself is so large that it would not be feasible to set up 532 transfection plates (76 library plates x 7 transfection plates) in one day. By using a strong control, a clear separation should be seen between the positive and negative controls, in each batch setup. Figure 3.13A depicts the raw data (absorbance) per plate that is also shown in Figure 3.13B (normalised). As can be seen, there is not always a clear separation between the positive controls (blue) and negative controls (red), as some data points clearly overlap (e.g. plate number 13). This may be a systemic error due to setup of transfection plates from different library plates. This is a common occurrence and normalisation steps have been developed to remove this effect from the generated data. This involves the placement of both negative and positive controls on each plate. By normalising the data to the median of the negative controls per plate, any systemic error is removed, and a clear separation is noted between negative and positive controls. This is visible in Figure 3.13B which depicts the data after it has been normalised to the median of the negative controls per plate.

A



B

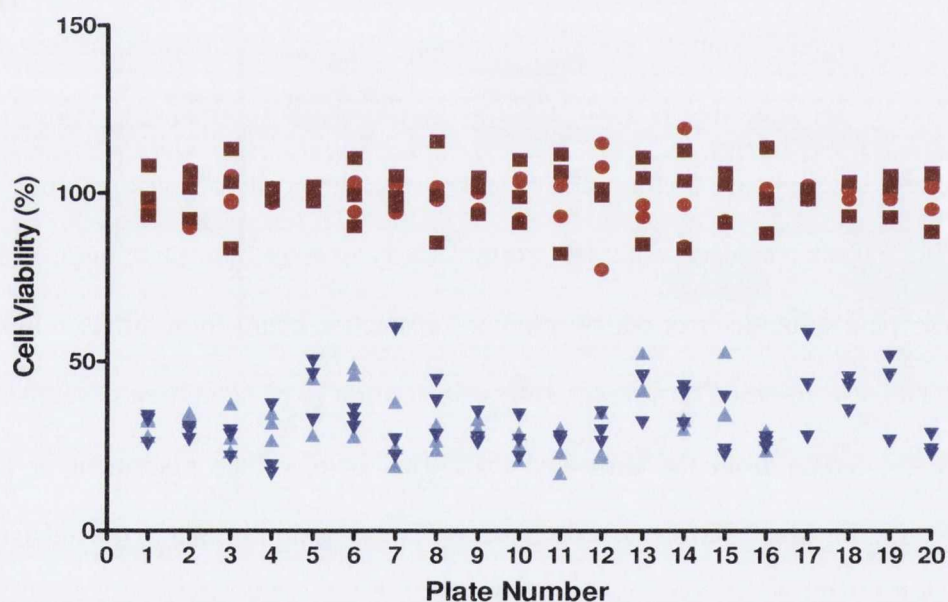


Figure 3.13: Plate-Well Series Plot of Pilot Screen Data. This graph shows raw data and values normalized to median of negative controls per plate. A) Graphical depiction of raw data (Absorbance @ 570nm) shows batch variation; B) The data from graph A, normalized to the median of the negative controls per plate shows reduced batch variation. N=3. Colours as used for labelling as in fig. 3.11B. (t-test; $p < 0.0001$)

3.3.4.2 Students t-Test

The unpaired student's t-test was used to analyse the significance of the reduction in cell viability as compared to the median of the negative control. The results in figure 3.12A & 3.12B suggest that there is a statistically significant difference between the positive controls as compared to the negative control as analysed using the unpaired t-test outlined in section 2.8.1. It was concluded, using the t-test, that both of the chosen siRNAs (GATA6 and Bcl-xL) would act as strong positive controls for use in the screen as the p-value obtained (Figure 3.12A) was $p < 0.0001$ indicating a highly significant reduction in cell viability.

3.3.4.3 Z-Factor

The criteria shown in Table 3.1 are the cut-off ranges used for determining the Z-Factor (section 2.8.1). The Z-factor is based on the standard deviation of the data, divided by the mean. Therefore, if one data-point is clearly different from the mean, increasing the standard deviation, the Z-factor will not be very low, and not classed as strong. For the pilot screen dataset given previously (Figure 3.12 & Figure 3.13), the Z-Factors are all classified as strong controls, with the exception of 2 data points as highlighted by a pink box (Figure 3.14A). The reason for these two plates being insignificant is due to the presence of an outlier in each plate which increased the standard deviation.

Table 3.1: Z-Factor Criteria for Classification of Assay Quality

Category	Z-Factor Criteria
Perfect	1
Extremely Strong Control	$1 > \text{Z-Factor} \geq 0.5$
Strong Control	$0.5 > \text{Z-Factor} > 0$
Not Good	0
Unusable	< 0

3.3.4.4Strictly Standardised Mean Difference (SSMD)

Like the Z-Factor, SSMD (section 2.8.1) also obeys a strict set of criteria (Table 3.2) to which results must adhere for determination of significance. The SSMD for quality control was calculated (Figure 3.14B), for the pilot screen dataset (Figure 3.12 & Figure 3.13) along with the Z-Factor (Figure 3.14A), to compare the results for the efficacy of the controls in each column.

The results, shown in Figure 3.14A & B, show that there is little or no difference between the amount of extremely strong controls shown in purple (Z-Factor=12, SSMD=13), while the main difference is seen in the strong controls shown in orange (Z-Factor=19, SSMD=8). The overall conclusion that can be drawn from these two graphs is that there are more strong controls, as defined by Table 3.2, seen in column 2 as compared to column 1. This result correlates with the conclusion drawn in section 3.3.3.1 and Figure 3.12B, that column 2 should be chosen for addition of controls.

Table 3.2: SSMD- Based QC Criteria when Using an Extremely Strong Control

Quality	SSMD Based QC Criteria
Excellent	$\hat{\beta} \leq -7$
Good	$-7 < \hat{\beta} \leq -5$
Inferior	$-5 < \hat{\beta} \leq -3$
Poor	$\hat{\beta} > -3$

$\hat{\beta}$: Denotes SSMD Value. The values are negative as these are used when the value of the positive controls is lower than that of the negative controls, therefore indicating a reduction in activity, or in this case, cell proliferation.

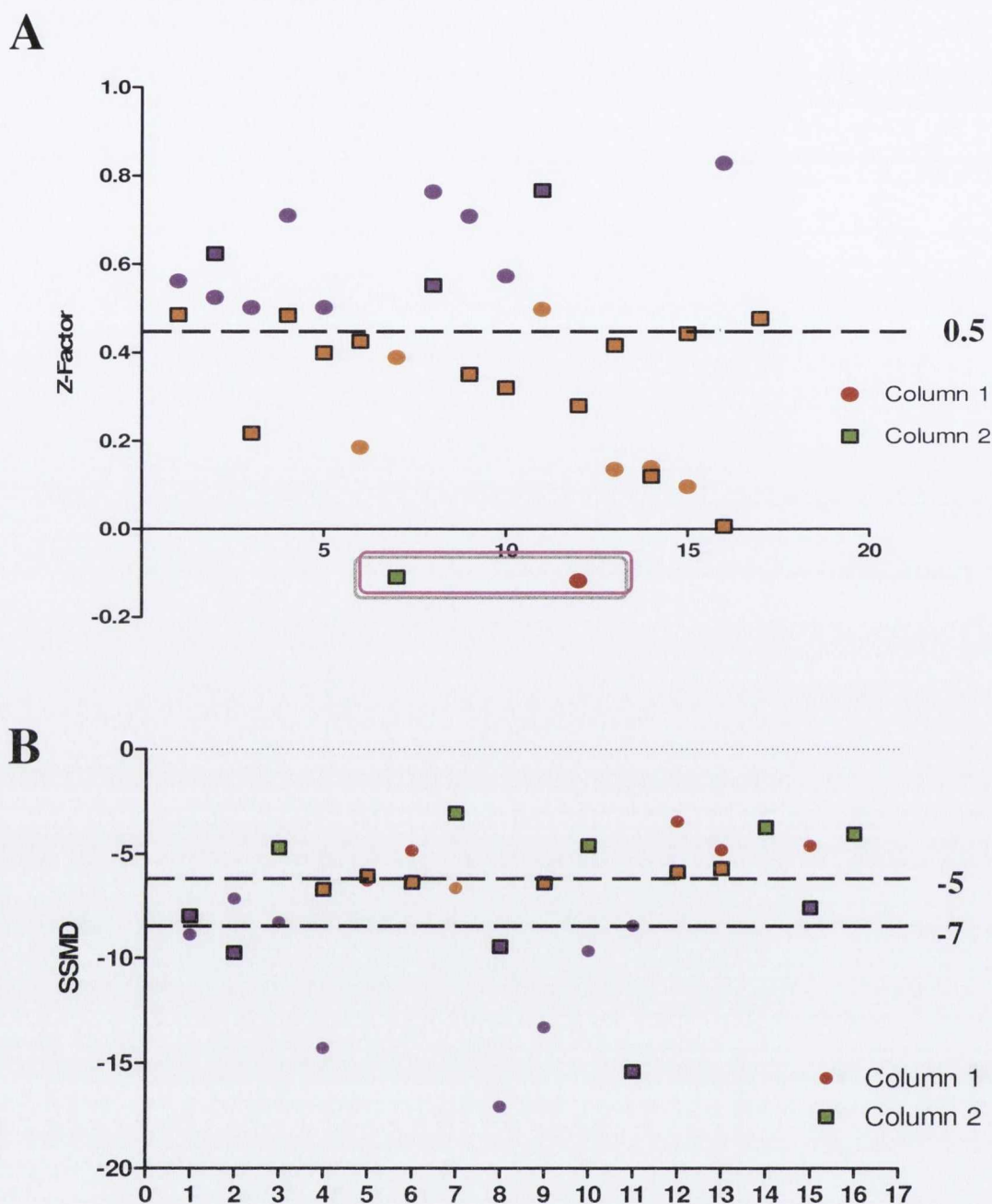


Figure 3.14: Figures Depicting Quality Control Statistics. Scatterplots represent pilot screen data also shown in fig 3.11 & 3.12. A) Graph represents Z-Factors calculated for pilot screen data. All data shown is deemed to be strong controls, except for 2 points (red and green). Dashed line represents cut-off for extremely strong control (0.5). Strong control (orange), extremely strong control (purple). B) Graphical representation of SSMD used for QC. All data except for 9 points deemed to be strong controls. N=3. Data is represented as non-targeting (negative control) as compared to either GATA6 (red) or Bcl-xL (green), both of which represent the potential positive controls. (Outliers noted and removed to prevent screwed data analysis)

3.4 Discussion

The overall aim of this body of work was to optimise all parameters leading to a successful large-scale siRNA library screen, limiting any possible variation that may arise due to batch-to-batch variances. Systemic screening involves silencing of individual genes in each well, arrayed within a 96- or 384-well plate. The challenge with this type of screening method is to make sure the assays are sensitive and robust enough to allow for reproducible results. It has been shown that optimization of this particular type of screen is significantly challenging and is usually underestimated (Echeverri and Perrimon, 2006). Sufficient optimization is also necessary to reduce risk of possible off-target effects, which is achieved via thorough design and implementation utilising efficient controls. This chapter has identified several steps which can minimise any possible variation and developed successful statistical analyses for determination of quality control and assurance.

Determining the correct cell seeding density is highly important, as the overall aim of the siRNA screen is to determine effect on cell viability after treatment over a period of time. Plating too many cells would result in over-confluent cells which would inhibit the transfection efficiency whereas seeding too few cells could result in false-positives, as too many cells would die. Identification of good positive and negative controls is necessary as they allow for the monitoring of the transfection efficiency and cytotoxicity on the cells, respectively. High transfection efficiencies are required in all experiments; if the transfection efficiency is low, this could result in reduced gene knockdown and phenotypic effects, along with a low level of reproducibility. A negative control should not affect the cells significantly when compared to untreated (resting) cells; therefore, for all experiments there should also be untreated cells.

Negative controls can help to identify any possible sequence-dependent off-target effects and should show consistent effect size across experiments to allow for adjustments due to any possible spatial effects. Pilot screens are also a necessary optimization step in the up-scaling of the experiment as they can detect any possible variation that may arise due to plates being setup in batches on separate days. They are used to optimize several parameters in parallel to achieve maximal transfection efficiency while keeping cytotoxicity levels low (Zhang, 2011b).

Data analysis of the screen output is as important as the screen setup itself. The first step in data analysis is to determine the quality of the screen (QA/QC) as a high-quality screen must be able to confidently identify compounds with significant biological activity. Statistical analysis of the pilot screen results determines the best tests to use and concludes if the pilot screen methodology is working as desired. There is a risk of systemic spatial effects such as evaporation of media liquid from edge wells of a plate, for this reason the data is normalised. Normalisation can be done on a plate-by-plate basis or on a whole experiment basis or if a screen is setup in such a way that we know a lot of true hits are on one particular plate, then it would be best to normalise the data on a whole experiment basis (Wiles et al., 2008). The t-statistic is a widely known and used statistical test that incorporates both sample size of the controls along with the degree of separation between both controls. It is a relatively simple test and quickly generates interpretable results. For this reason, P-values are given for all figures within this body of work indicating the common use of this test.

The Z-factor is a commonly used statistic in RNAi experiments for determination of screen quality and directly measures the sum of the standard deviations within the two controls. As previously outlined, pilot screens are necessary to determine the degree of separation between the negative control and positive control, to assess the reproducibility and signal variation at the two extremes of the activity range. Thus, the difference between the mean of the negative and positive controls is indicative of the dynamic range of the assay signal. The purpose of the Z-factor is to capture data variability within the controls of the dataset, and has been shown to be both accurate and precise. The Z-factor is sensitive to not only the assay procedure but also to the concentration of the compounds used, which will give different dynamic ranges and has thus been highlighted as a necessary parameter for assay optimisation.

The successful implementation of the Z-factor is demonstrated by the wide usage of this metric in RNAi screens. Several recent studies have supported the continued use of the Z-factor since its development in 1999 by Zhang et al. (Goel et al., 2014, Rajamuthiah et al., 2014, Crowther et al., 2014, Li et al., 2013b). Each of these studies highlights the utility of the Z-factor for quality control, and for each, the Z-factor values were above 0.5, indicating a successful screen. The study by Goel et al., was setup to identify modulators of smooth muscle cell proliferation and used both the Z-factor and the reduction in cell viability between the negative and positive controls as indicators of screen quality (Goel et al., 2014). Another recent study involved a whole genome siRNA screen to identify regulators of NOD2 and NF- κ B signalling in Hek293 cells, using the Z-factor to assess assay quality and robustness. The experiment indicated that the majority of the 204 plates screened, had Z-factor values above 0.5 which is considered good, although the percentage of plates above 0.5 was

not given. This study evaluated the Z-factor using two different assays; NF- κ B luciferase and cell viability using CellTiter Fluor assay, and was determined per plate, for each assay, with both assays revealing the same results (Warner et al., 2013).

Another statistical test, the *strictly standardised mean difference* (SSMD), differs from the Z-factor as it utilises the standard deviation of the difference between the two controls (Zhang, 2011b). It has been described as an accurate but '*less conservative*' gauge of the quality of a screen (Birmingham et al., 2009) and is based on the probability that a value within the positive controls will be greater than a value in the negative controls. In our experiment we are looking for a decrease in cell viability, therefore, the probability that the positive controls will be less than the negative controls, given this, the results are denoted as negative values (e.g. -5). The cut-off values for SSMD have been pre-set by (Zhang, 2011b), and also summarized in Table 3.2. The success of SSMD for quality control has been shown in recent studies including the development of a phenotypic screen to analyse the effect of antimalarial drugs. The screen in this study used the SSMD for quality control with an overall SSMD value of 2.62, which indicated a highly successful screen (Lee et al., 2014).

Although several studies are published using the above parameters, many studies still simply rely on the t-test and post-hoc Mann-Whitney or similar metrics. For example PLK1, CDK1 and NDC80 have been identified as potential targets for malignant pleural mesothelioma treatment. This screen was setup *in vitro*, and a panel 40 siRNAs were analysed. The main issue with this study is that there is no published quality control statistics for this study other than the identification that growth was

inhibited by 50% by 7 siRNAs. These 7 siRNAs were then taken, and knockdown quality was confirmed by analysis of mRNA levels (Linton et al., 2014). Another example of a lack of sufficient statistical analysis is demonstrated upon the identification of TNK1 in the growth and survival of pancreatic cells. This study used a decrease in cell viability as compared to the negative control, as an indication of transfection success, while the data was normalised using the z-score method (Henderson et al., 2011). This indicates a lack of sufficient quality control metrics such as the t-test, Z-factor or SSMD, instead simply relying on a decrease in cell viability. The risk associated with this is the false identification of a 'successful' screen due to the presence of false positives which could be due to high toxicity levels for example. A possible reason that sufficient quality metrics were not performed may be due to the complex nature of statistical tests such as the Z-factor and SSMD, but these are now incorporated into many statistical software packages available, including R. This begs the question, how reliable are the results being documented if sufficient quality control metrics are not be analysed which are specifically designed for RNAi screen analysis?

For data analysis within this body of work, it was decided to utilise multiple overlapping statistical tests; namely t-statistic, Z-Factor and SSMD. Each test benefits the researcher in different ways; t-test is beneficial for determining mean difference between the negative and positive controls, the Z-factor is beneficial as it takes data variability into account and the SSMD is beneficial as it accounts for data variability but uses a probability based method. If, by combining the results of all of these metrics, we come out with good quality control results, then we can be highly confident that the screen is a success, although the combination of these quality

control metrics has not been utilised by many studies. As previously identified most studies will only use the t-test and possibly one other statistic, most commonly the Z-factor, for assessment of quality. Given this, in one recent study both parameters, Z-factor and SSMD, were utilised to check the quality of results following the development of an automated method for image analysis (Talati et al.). This proposes the diversity of the SMMD for quality control, along with the confidence that researchers hold in the parameter.

As previously stated, off-target effects are a major issue in RNAi screening and the use of siRNA pools helps reduce the concentration of siRNA necessary for efficient transfection, and thus toxicity levels; but there are also other methods used to reduce off-target effects. Utilisation of bioinformatics software such as BLAST, which is used to analyse sequence specificity, has also been shown to reduce the risk of off-target effects. This method can be used to input the known siRNA sequences supplied when designing a siRNA, which will then align them and determine which genes contain the same sequence, or portion of the sequence. Specificity is thus determined if there is 100% alignment shown for the intended gene alone. With commercially available siRNA the risk of this OTE is now minimal due to continual removal of these types of sequences (Sharma and Rao, 2009). Finally chemical modifications to siRNAs are also necessary to reduce the risk of off-target effects. There are different types for different issues that can target OTEs and stability. Jackson et al., identified a number of sequence-independent modifications that can be implemented for this reason; Table 3.3 indicates the possible modifications and why they are used. All of these principles have been identified by Dharmacon and have been incorporated into their ON-TARGETplus and SMARTpool siRNA reagents. Conversely these have

been used throughout this body of work; the siRNA library screen used SMARTpool siRNA reagents while subsequent verification and validation utilises ON-TARGETplus siRNA reagents.

Table 3.3: Chemical Modifications for Stability and Specificity of siRNAs

	Modification	Why Are These Necessary?
Sense Strand Disabling	2'-O-fluoro	Increase sequence space for siRNA design and activity
	2'-O-alkyl	
Stabilisation	2'-halogen	For stabilisation of siRNAs in biological fluids
	2'-alkyl	
	2'-O-alkyl	
Specificity	2'-O-alkyl	Impedes strand entry to RISC to minimises sense and antisense OTEs

The siRNA SMARTpool reagents supplied by Dharmacon, which contain a pool of 4 sequences for each individual gene, were used for all analysis within this chapter, while bioinformatics software has been used to determine the specificity of the sequences. The suppliers have also indicated that chemical modifications have been applied but have not identified the specific modification (ThermoScientific, 2013). Combining this information with the stringent QA/QC statistical analyses implemented we can be confident that our results are real and specific.

In conclusion, this body of work has succeeded in optimizing all necessary parameters for successful siRNA library screen setup to analyse effect on cell proliferation. These experiments should ultimately be repeated for other cell lines, but overall each test can be adapted to suit the cell type used. The statistical analyses outlined above have been reported already (Zhang, 2011b, Zhang, 2011a) but by using the combination of all 3 tests; t-test, Z-Factor and SSMD, screening quality can be ascertained with very high confidence. These optimisation steps were undertaken to analyse effect on cell proliferation following siRNA treatment but another possibility for analysis could be to treat transfection plates with a bile acid such as deoxycholic acid (DCA). This treatment in combination with the siRNA knockdown may identify a different set of gene hits that could be followed up separately. Another possible step would be to fix a siRNA treated plate to analyse for effect on cell morphology using actin, tubulin and Hoechst antibodies thus investigating the effect of siRNA knockdown on cell motility. Overall, the success of this body of work is important in the development of the screen which will ultimately be setup to identify novel genes that can regulate oesophageal cell proliferation.

Chapter 4

Results

*Implementation of a Druggable
Genome siRNA Library Screen to
Identify Novel Genes Regulating OAC
Cell Proliferation*

4 siRNA Screen Implementation and Analysis

4.1 Introduction

In 2006, the Nobel Prize in Physiology or Medicine was awarded to Andrew Fire and Craig Mello for their discovery of RNA interference, in *C. elegans* and *A. thaliana*. They both showed that, in two different systems, sequence-specific post-transcriptional gene silencing utilising the RNA-induced silencing complex (RISC) degraded mRNA and could hence silence gene expression. (Figure 4.1) (Fire et al., 1998b, Mello and Conte, 2004). This discovery coincided with the publication of the *C.elegans* genome sequence followed by subsequent publication of the *D. melanogaster* and human genome sequences. The combination of these discoveries has resulted in advances in mammalian cell research as the RNAi mechanism identified in *C.elegans* and *A Thaliana* has been shown to be conserved in mammalian cells. Furthermore, this work has led to a change in how biomedical genetic research is undertaken; by silencing specific genes we can now potentially identify their functions and hence their potential roles in disease. It is also possible therefore to identify therapeutic targets and to ultimately consider the use of inhibitory RNAs as potential therapeutics in their own right.

As previously described, gene interference is initiated upon cleavage of double stranded RNA (dsRNA) by Dicer, which is a RNA III enzyme, producing small interfering RNA (siRNA). A dsRNA-binding protein then recruits this Dicer-siRNA complex to Ago-2 which acts to cleave the anti-guide strand of the siRNA duplex. Subsequent cleavage of the passenger strand results in assembly of the RISC complex containing Dicer, single strand RNA (ssRNA) and Ago2. The ssRNA can then guide

cleavage of the target RNA by Ago2 (Agrawal et al., 2003, Mello and Conte, 2004) (Figure 4.1).

The main difference noted between *A. thaliana* (plant), *D. melanogaster* (arthropod), *C.elegans* (nematode) and humans (mammal), is the Dicer enzyme that cleaves the long dsRNA. Humans and *C. elegans* have been shown to only express one isoform of the Dicer enzyme, while *D. melanogaster* and *A. thaliana* are shown to express two and four Dicer-like proteins (DCLs) respectively (Lee et al., 2004, Xie et al., 2004). Knockout studies in both of these species support the notion that there may be a functional redundancy amongst these DCL paralogues (Gasciolli et al., 2005, Lee et al., 2004), which may also be true for the RISC component Argonaute. Within this family, there are four Argonaute proteins expressed in humans, but Ago2 is the only member of this specific protein family that has a slicer function therefore mediating cleavage of miRNA and siRNA (Czech and Hannon, 2011). Eukaryotes, on the other hand appear to use multiple members of the Argonaut family, with *A. thaliana* expressing 10 members and *D. melanogaster* expressing 5 (Hunter et al., 2003, Williams and Rubin, 2002).

Following from the concurrent publications of both the discovery of RNAi and the genomes of different species, genome wide high-throughput RNAi screens were being published within the *C. elegans* research field, which was closely followed by *D. melanogaster* and mammals. In *C.elegans*, a whole genome RNAi screen aiming to identify gene function by systematic gene knockdown identified 24 genes believed to play a role in germline development (Maeda et al., 2001). In *D.melanogaster*, a systematic RNAi screen was utilised to determine the functional roles of kinases and

phosphatases within *D. melanogaster* to investigate the signalling response to Hedgehog (Hh). Two genes were identified, Dally-like protein and casein kinase alpha-1, as possible Hh pathway components (Lum et al., 2003). One example of a murine study utilised a shRNA library to identify components affecting p53-dependent proliferation arrest in transformed cells. This work identified 5 novel genes and 1 known gene whose suppression results in resistance to p53-dependent proliferation arrest, and elimination of DNA-damage-induced G1 cell-cycle arrest (Berns et al., 2004).

This progression of study in different phyla has led to the wide use of RNAi screens, and the commercial development of RNAi libraries for genome screening. Genome-wide screens have been highly successful in the identification of specific gene functions. The creation of siRNA libraries arrayed in 96- and 384-well formats, has resulted in the utilisation of synthetically made siRNAs in large scale screens. The first published account of siRNA library use within humans, was in 2007, when Whitehurst *et al.* utilised a large library resulting in the identification of 87 genes that sensitised a lung cancer cell line to paclitaxel (Whitehurst et al., 2007). Following this further screens were published which analysed the effect of specific gene silencing on cell viability, cell cycle and invasive potential. Such studies have included the identification of KAT8 as a regulator of lung cancer cell viability in A549 cells following a loss of function RNAi screen targeting 32 genes (Zhang et al., 2013b). This study suggested that inhibition of KAT8 can lead to p53 induction and subsequent reduction in bcl-2 expression, therefore reducing the lung cancer cell growth. Another study utilised a kinome library containing 779 siRNA pools, with the aim of identifying a novel therapeutic target that can enhance taxane effects by

inhibition of mitosis while continuing to provide taxane- and mitosis-independent mechanisms of ovarian cancer cell death. It identified SIK2 as playing a key role in mitosis initiation as its inhibition significantly delayed mitotic progression in ovarian cancer cells (Ahmed et al., 2010). In other screens, a large number of genes have been identified that regulate cell migration in breast cancer cell lines MCF-10A, including MYLK and ACP5 which induced enlarged protrusions and nuclear rotation respectively (Simpson et al., 2008). These studies highlight the diverse range of cellular processes that can be analysed using RNAi screens.

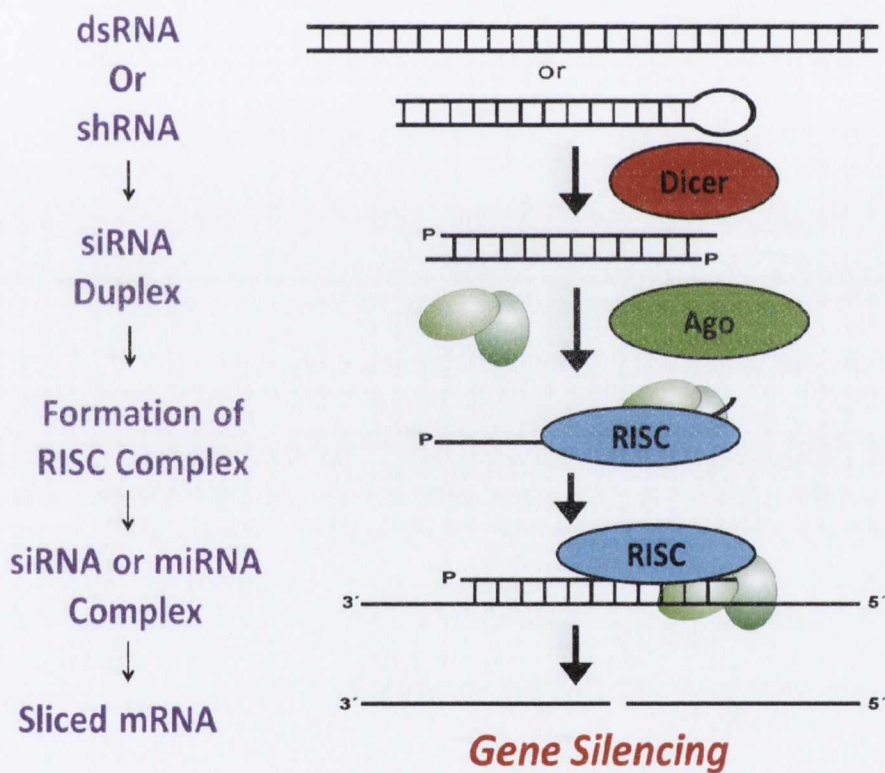


Figure 4.1: RNA Interference Resulting in Gene Silencing. Dicer cleaves dsRNA into a siRNA duplex. Argonaute (Ago) is recruited which is the main component of RISC, in conjunction with Dicer and TRBP. The RISC complex then acts to unwind the dsRNA resulting in subsequent gene silencing, by mRNA degradation.

RNAi screening offers us the ability to determine gene function and identify possible gene targets which would allow the development of specific therapeutics (Jackson and Linsley, 2010). As previously outlined, there are two main types of RNAi constructs that can be utilised for mediated gene knockdown; small interfering RNAs (siRNA) and short hairpin RNAs (shRNA). These two molecules differ largely in one respect; siRNA is a chemically synthesised dsRNA, while shRNA is vector-based (Figure 4.2). The shRNA consists of two complementary RNA sequences which are linked by a short loop which is comparable to the hairpin found in naturally occurring miRNA. Following transcription, the shRNA sequence is transferred to the cytosol where it can be recognized by Dicer, which processes the shRNA into the siRNA duplexes (Moore et al., 2010). However, siRNAs can be chemically synthesised or produced *in vivo* or *in vitro* facilitating delivery of the siRNA duplex directly to the cytosol. Efficient shRNA transfection requires delivery of the lentiviral shRNA to the cell which is then produced internally from a DNA construct which encodes the sequences for ssRNA. shRNA are preferable for the stable transfection of cells, but for our work we only required transient transfection, therefore the use of siRNA requires less time, and generates minimal cellular toxicity. They are also preferential for therapeutic use as they do not require the utilisation of vectors as shRNA do (Burnett et al., 2011). For these reasons a siRNA library was utilised for this body of work.

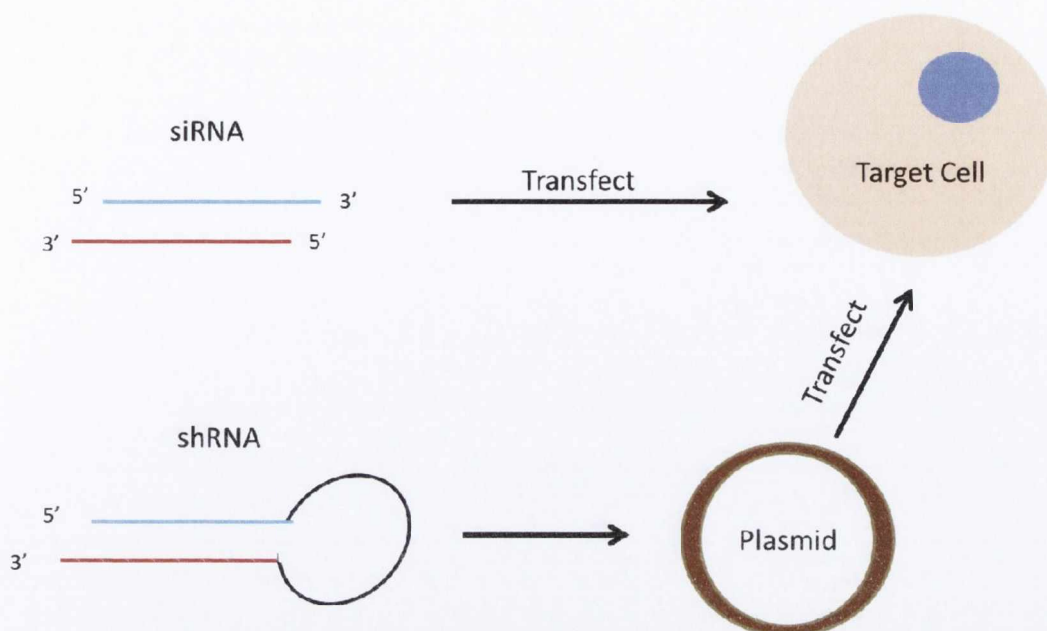


Figure 4.2: Transfection with siRNA Differs from shRNA. siRNA (19-22nt in length) can be directly transfected into a target cell. shRNA contains a short hairpin loop of 4-11nt in length and requires construction in a plasmid backbone which can then be transfected into the target cell.

High-throughput technologies such as large-scale siRNA library screens play a central role in molecular biology and drug discovery. They allow for the simultaneous analysis of thousands of genes, with the intention of identifying previously unknown genes involved in a specific disease or pathway, with the potential to lead to development of novel drugs. High-throughput screens can identify pathways that, when inhibited, can re-sensitise drug-resistant cells to a specific drug. For example a synthetic lethal screen found that inhibiting either the NF κ B pathway, IKBKB gene or NIBP gene using an IKBKB inhibitor, can re-sensitise lapatinib-resistant breast cancer cell lines to sub-lethal doses of lapatinib (Wetterskog et al., 2013). On the other hand, an RNAi screen, if not performed and analysed correctly can result in the production of misleading results. For example, one study in particular highlighted the risks involved in using a siRNA screen that is not properly designed. This study

aimed to identify modulators of the TGF- β pathway via confocal imaging in the HaCaT keratinocyte cell line. The library used was specifically designed, and contained 6,000 individual siRNAs targeting genes with a known function in cancer development. This study used a fluorescent microscopy nuclear translocation assay that consisted of a GFP-SMAD2 fusion protein stably expressed in the HaCaT cells. Results indicated that all identified hits were off-target effects, as highlighted by the fact that 20% of the hits found are known not to be expressed in the HaCaT cell line (Schultz et al., 2011). The group has highlighted that the OTEs observed may be due to both the specific assay used and the siRNA library itself. It was also noted that translocation was affected by cell density, especially in wells at the edge of the plates as compared to the control wells in the centre of the plates. Data was normalised to the cell number to correct for this, but validation experiments could not reproduce the results. While this study had 3 individual siRNAs targeting a single gene, these were plated separately rather than in pools. There were no multiple siRNA hits for the same gene possibly due to low knockdown efficiency in the cell line. This study highlights the need for rigorous design of siRNA screens, with particular attention paid to variables such as cell density, the design of the siRNA sequences and the use of pooled sequences to minimise off target effects and optimise transfection efficiency.

The use of genome-wide RNAi library screens has also helped to highlight many previously unknown genes involved in the reduction of cancer cell viability. For example, a recent study has identified two new androgen-receptor regulators, MED19 and HIPK2, which have been shown to reduce castration-resistant prostate cancer cell viability ($p < 0.05$) (Imberg-Kazdan et al., 2013). Furthermore, siRNA mediated inhibition of PLK1 can reduce triple-negative breast cancer cell growth by 80% at

72hrs post-transfection (Hu et al., 2012). A siRNA library screen has never been published in oesophageal adenocarcinoma research; therefore the druggable genome siRNA library screen to be implemented within this body of work is unique. The success of the above studies in both prostate and breast cancers indicate that, if successful, the genes identified could pave the way for design of approaches to novel molecular therapeutics for the treatment of oesophageal cancer.

The main aim of an RNAi screen is to select hits with the most desired effect (inhibition or activation); therefore the generated data needs to be effectively statistically analysed to identify both the quality of the screen and also to identify genes that give rise to the desired effect, known as hits (hit selection). The first step is to identify the tests and subsequent equations to be used for this analysis, as screens without replicates require different equations for some tests compared to screens with replicates. After identification of the necessary steps, they must be utilised to determine the quality and success of the screen, by comparing the separation band between a known positive and negative control (Zhang, 2007). If it is concluded that the screen was successful, then one can progress to selection of hits from the dataset. Statistical tests have been created especially for the purpose of RNAi screen analysis (Zhang et al., 2010, Zhang, 2011a), and these methods have now been incorporated into software such as R statistical software (Rieber et al., 2009, Pelz et al., 2010) to facilitate analysis of large datasets which can be analysed at a relatively fast pace, as compared to manual analysis. R is both a software language and environment that allows for manipulation, calculation and graphical display of large datasets. The R software can also be extended to incorporate more statistical tests using packages that

are freely available online via CRAN (Comprehensive R Archive Network), including CellHTS2 and RNAiR which are commonly used in RNAi screens.

4.2 Hypothesis and Objectives

So the question is thus asked, can a large scale siRNA screen be successfully implemented using the parameters optimised in chapter 3 to identify genes involved in oesophageal cell viability? This chapter aims to answer this question by setting up a large scale siRNA screen to identify genes, which when silenced, can significantly reduce oesophageal cancer cell viability. Statistical and bio-informatic analyses was undertaken to identify '*high confidence hits*' which could be brought forward for validation. These aims were achieved through:

- Implementation of a large scale druggable genome siRNA library screen, which contains 6022 individual siRNA gene targets using an end-point MTT cell viability assay
- Statistical analysis of the results of the screen utilising R software platform, utilising two specific packages, CellHTS2 and RNAiR, to identify '*high confidence hits*'
- Utilise bioinformatics software IPA and DAVID to ascertain genes that are functionally important
- Identify gene targets to be validated and verify the knockdown in oesophageal cell lines spanning the carcinogenic sequence

4.3 Results

4.3.1 Screen Implementation

The druggable genome contains siRNAs targeting genes that are known to bind drug-like molecules. The principle behind this siRNA screen was to identify genes which, when transcriptionally silenced via siRNA, would indicate a statistically significant reduction in cell viability after 96hrs. This would therefore highlight genes necessary for the growth of the high-grade dysplastic cell line used (GOhTRT) which has previously been shown to be resistant to DCA induced apoptosis. Optimisation steps with the GOhTRT cell line were highly reproducible, showing minimal variation, as compared to other OAC cell lines, when using the end-point colorimetric MTT cell viability assay.

To capitalise on the setup of the screen, other metrics were included; by setting up two different arms of analysis (Figure 2.5), a larger number of '*hits*' would be generated. The initial aim was to solely analyse the effect of silencing the 6022 genes on cell viability; which encompasses the first arm of analysis. For the second arm of analysis, effect on cell morphology following gene inhibition would be undertaken by fixing the cells and immunofluorescent staining for structural proteins including actin and tubulin. Analysis of the data generated in this arm is currently underway by another member of the lab to better understand the effect on metastasis and invasive potential.

4.3.2 Screen Setup

Drawing from the conclusions drawn in chapter 3, 5×10^3 cells per well were seeded in 96-well plates using the Matrix Wellmate robot. Simultaneously, the transfection reagent, DharmaFECT4, was made up using a volume of $0.2 \mu\text{L}$ per well; and the ready-made daughter plates were thawed. Each component, siRNA and transfection reagent, were added at specific time points and allowed to complex, then added to the pre-plated cells. All transfection plates were then incubated for 93 hours post transfection. MTT was added ($10 \mu\text{L}$) to each well, then incubated for a further 3 hours before addition of the detergent reagent ($100 \mu\text{L}$).

After the completion of the MTT assay, plates were read on a spectrophotometer at 570nm. The data generated from the screen was sorted, organised, and run through R statistical software for analysis to determine statistically significant hits. A sample of the computer code used for the analysis and generation of an interpretable output file is shown in the appendix 8.2.

4.3.3 Statistical Analysis of Screen Output

As described previously, the R statistical software system was utilised to run two programmes, CellHTS2 and RNAither, in order to help analyse the large amount of data generated from the screen (section 2.8). This software is used for statistical computing and graphics. There are a number of different steps to be undertaken before establishment of the final hit list as outlined in Figure 2.7. If the screen has been deemed to be of a high quality after the quality control (QC) analysis, then further statistical tests are run to identify the top hits, which in this case are the genes

that, when silenced using a corresponding siRNA, have maximal impact on cell viability.

4.3.3.1 Quality Control

Quality control statistical analysis determines, using specific statistical tests, if the quality of the screen was above or below standard. Normalisation can help identify outliers, and reduces any bias; the Z-factor determines the separation between the negative and positive controls, while the SSMD is based on the probability that the mean of one group (e.g. negative control) will be larger than the mean of another group (e.g. positive control) e.g. $P(X > Y)$.

4.3.3.1.1 Normalisation

The first step was to normalise the data to the median of the negative controls, per plate, as outlined previously (section 3.3.4.1), the results of which can be seen in Figure 4.3. Identifying outliers in a large scale RNAi screen should be relatively simple, this is due to the fact that we use replicates ($N=3$) of a known sample with a known absorbance/output value (Malo et al., 2006). In the case of this screen, two clear outlier plates were noted, plates 22 and 75 (Figure 4.3A), therefore for all subsequent analysis these two plates were removed. Plate 22 was removed as two of the negative control wells in replicate 1 were infected, and plate 75 was removed as there was no transfection reagent (DharmaFECT4) added to the control wells. This information was noted before analysis had taken place, therefore there was just reason to remove them from any further analysis. These plates remain in Figure 4.3A simply for the purpose of demonstrating outliers. Figure 4.3B is a boxplot indicating the

spread of the normalized absorbance values of the negative and positive controls relative to the samples.

4.3.3.1.2 Z-Factor

Each graph in Figure 4.4 indicates the control data for each replicate plate from the screen and the Z-factor value is shown at the top of each graph. A Z-factor of between 0 and 0.5 is considered good (table 3.1), therefore we can conclude that the screen was successful and usable, allowing for the continuation of our analysis.

4.3.3.1.3 Strictly Standardised Mean Difference (SSMD)

SSMD for quality control, as described previously (section 3.3.4.4), uses standard deviation rather than the sum of the controls. Figure 4.5 shows the SSMD values for the screen, in relation to the quality control. The cut-off values of -5 and -7, as indicated, were set using the guidelines set out in table 3.2. As can be seen, only 14 plates out of 228 were below the SSMD cut-off of -5 indicating that 6% are above the -5 cut-off and 23% are above the -7 cut-off value. The conclusion drawn from this is that the SSMD data, using a cut-off of -5, combined with the Z-factor results indicate a successful screen.

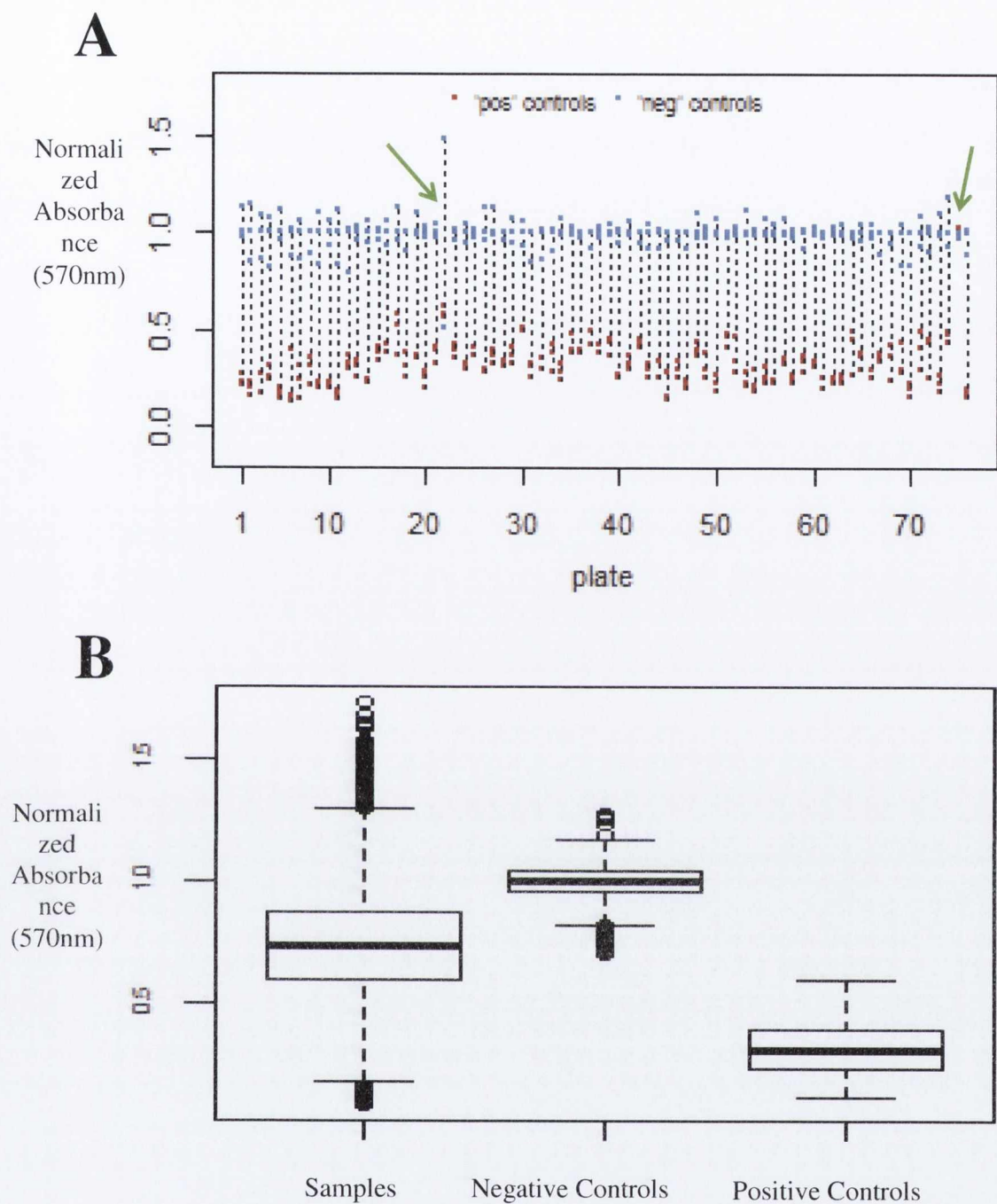


Figure 4.3: Normalization of Control Raw Values to the Median of the Negative Controls per Plate. A) Data shown is for replicate 1, for all 76 plates only. Controls are normalized using median of the negative controls. Plates 22 and 75 (green arrows) are clear outliers, and are therefore removed for all further analysis. Positive Controls (Red); Negative Controls (Blue). Generated using CellHTS2; B) Boxplot showing separation between samples, negative controls and positive controls (generated using RNAither, with plates 22 and 75 excluded). N=1.

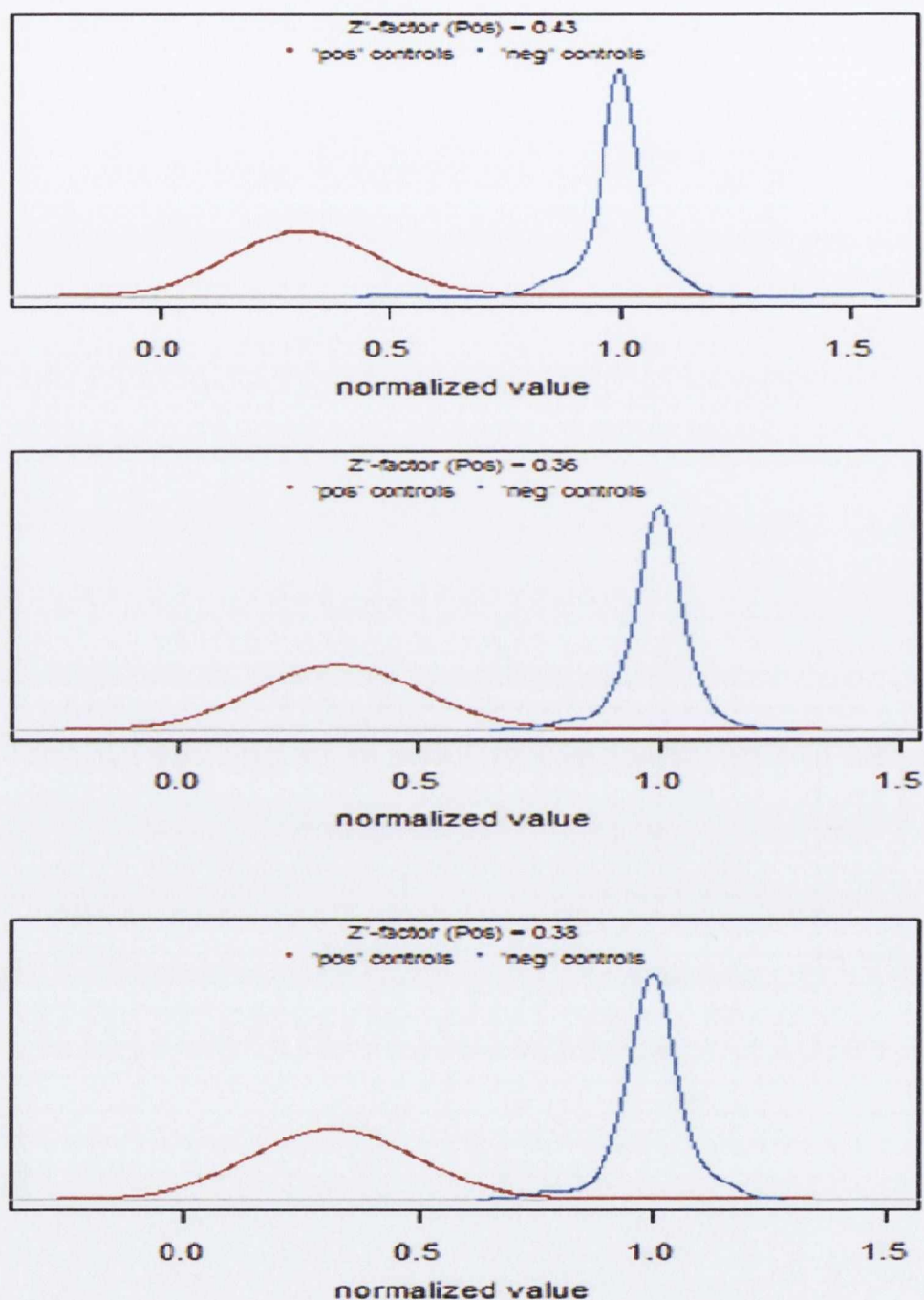


Figure 4.4: Z-Factor Graphs for Each Replicate Incorporating all Control Wells from 76 Screen Plates. The line charts above represent the control wells for all 76 screen plates, separated by replicate number. The Z-factor values for each replicate are 0.43, 0.36 and 0.33 respectively. As all values are between 0 and 0.5 they are considered good. All graphs show a good separating between the positive (red) and negative (blue) controls. Graphs and data were generated by CellHTS2 package used through R statistical software. N=1.

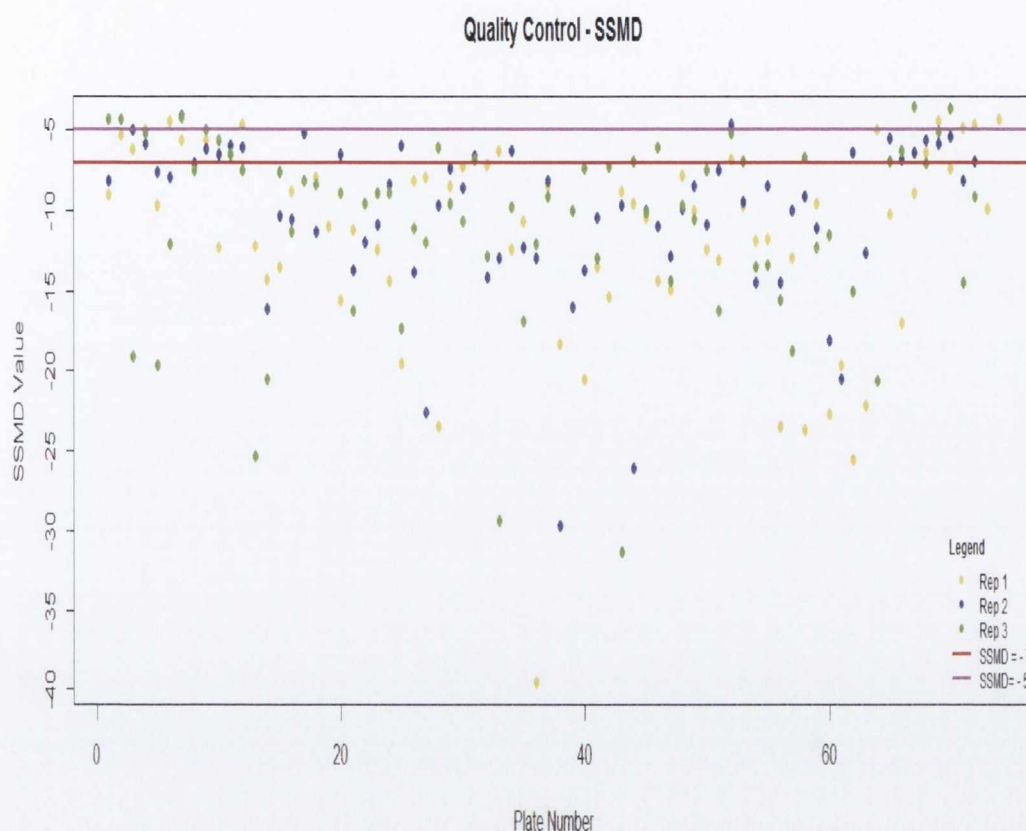


Figure 4.5: SSMD Quality Control Values for Screen Data. SSMD quality control values for each replicate, emphasising that 14 plates are above a cut-off value of -5, and 53 are above a cut-off value of -7. N=1. These results indicate a good quality screen. (Graph supplied by Dr. Fiona Behan)

4.3.3.2 Hit Selection

The main goal of this large scale screen is to identify ‘hits’, which are siRNAs that show the pre-defined level of reduction in cell viability (section 2.8.2). This was undertaken by identifying and selecting the siRNAs with the largest effect on cell viability which could then be carried forward for validation. The statistical tests and cut-off values have previously been discussed in section 2.8.2. Four statistical tests were utilised for hit selection; t-test, percentage cell viability, z-score and SSMD.

The t-test was used to analyse the mean difference between the median of the negative controls per plate, and each sample per plate. This results in the commonly used output of the *p-value* which indicates whether the decrease in cell viability is significantly different from the mean of the negative control. The percentage cell viability represents the median of the triplicate samples wells as a percentage of the median of the negative control wells. This results in the cell viability of the samples being represented as a percentage of the negative controls. Following this test, we calculated that there were 1818 siRNAs with a p-value less than 0.01 which represents 30% of the total siRNAs within the druggable genome library.

The next statistical test chosen to help elucidate our hits was the “robust Z-Score” which is commonly used within RNAi screen analysis (Ooi et al., 2013, Birmingham et al., 2009, Zhang, 2011a). It is effective at identifying weaker hits within the data, along with controlling false positives and negatives, more so than the normal Z-Score which uses mean $\pm$ SD (Birmingham et al., 2009, Chung et al., 2008). Within a screen the majority of siRNAs will not show specific silencing effects, therefore true hits should behave like outliers. Given this information, the robust Z-Score estimates the

centre and variability of the data, while being more robust to outliers, utilising both the median and median of absolute deviation (MAD) for its calculation. The constant, 1.4826 is used to equate MAD to standard deviation (SD), when the measured values are normally distributed (Zhang, 2011b). As with the normal Z-Score which uses $\text{mean} \pm \text{SD}$, the number of standard deviations away from the mean is usually set to 2 or 3. Results showed that there were 250 siRNAs with a robust Z-Score of less than -1.6. This value is equivalent to a p-value of 0.05, which is the 95th percentile, therefore this value was chosen as an appropriate cut-off for our results. This use of different Z-score and p-value has recently been demonstrated, with the use of Z-score cut-off -1.5 and p-value cut-off 0.025, which are not equivalent (Stanley et al., 2014). It is not stipulated that the p-value should be equivalent to the Z-score for this type of analysis; therefore the Z-score cut-off was set at -1.6, as this still retains statistical significance.

To control for off-target effects within RNAi screens, a relatively new statistical method, Strictly Standardised Mean Difference (SSMD) for hit selection, has been developed (Zhang et al., 2010). Within RNAi screens the aim is to determine the size of the effect of the siRNA, whether it be activation or inhibition. Given this, the SSMD has been designed to take into account both sample size and siRNA effect size. This differs from the t-test which uses the hypothesis of no mean difference between two groups to measure statistical significance, but is similar to the Z-score as it takes into account variability in both groups being compared. It is also robust to the strength of the positive controls, while taking into account the probability of a hit being less than a negative control (Zhang, 2011b). Our results identified 903 siRNAs that

generated an SSMD value less than the standard cut-off of -5 (Figure 4.6 & Figure 4.7).

4.3.3.2.1 Graphical Depiction of Statistical Results

The percentage of siRNAs within each cut-off, compared to the total number of siRNAs in the screen are varied; 15% of the siRNAs are within the SSMD cut-off of less than -5, 30% are within the P-value cut-off of less than 0.01, 4.35% are within the cell viability cut-off of less than 40%, while the smallest number was found within the Z-Score cut-off of less than -1.6, with only 4.15% of siRNAs being contained within this cut-off. These values show how stringent the significance levels were set for all four tests, resulting in identification of 118 high confidence hits which are significant across this stringent set of parameters. Figure 4.6 shows the scatterplots for each statistical test, with the significant siRNAs enclosed in a red circle. While we have only included genes which are significant across all parameters in our list, we cannot exclude the possibility that genes in any individual list may also be functionally significant. Appendix 8.5 contains all genes within these cut-offs, with a total number of 1031 genes identified.

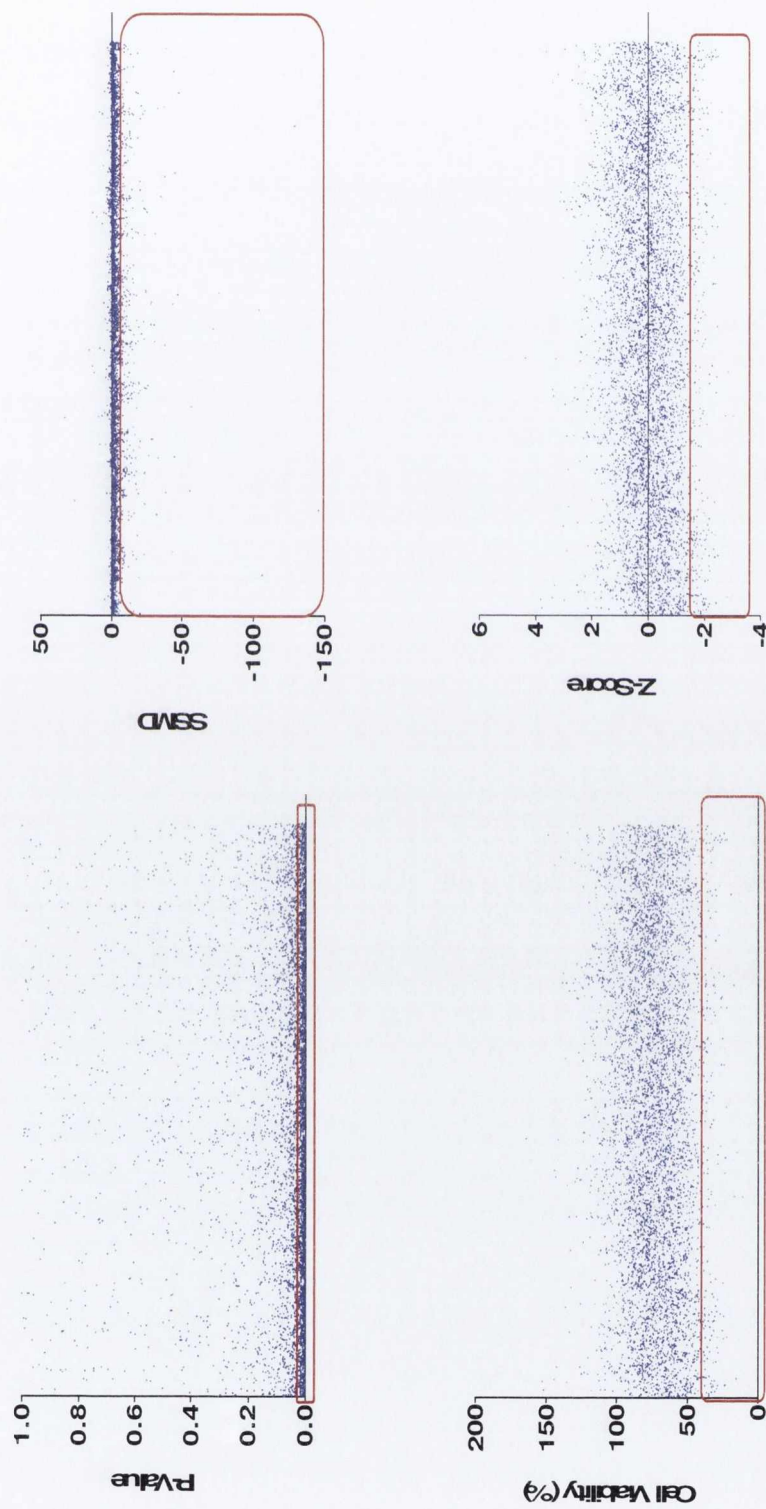


Figure 4.6: Scatterplots of All Data with Cut-Off Values. Scatterplots of complete dataset after removal of outliers, controls, and empty wells. Data is also represented in fig 24 below. Cut-off values for each test are indicated by the red line, with the data of importance encircled: SSMD < -5; Cell Viability < 40%; P-Value < 0.01; Z-Score < -1.6. N=1.

4.3.3.2.2 Identification of High Confidence Hits

Figure 4.7 shows the 4 statistical tests used, and the cut-off values set for each (as per table 2.3), highlighting the 118 high confidence hits that are common to all statistical tests. These siRNAs were then examined using several informatic pathway and gene ontology programmes (Figure 4.9) to help identify possible interesting candidate genes to carry forward for verification and validation.

Table 4.1 shows the 118 high confidence hits, the median raw value, normalised and Z-score values, along with the corresponding P-value, SSMD, cell viability and 100-cell viability. These genes have been ranked by median Z-score (column 5 in Bold). The two genes that we brought forward for subsequent validation following hit verification, LIF and C1QA, are also indicated in red.

Figure 4.8 is a graphical depiction of all 6022 genes within the druggable genome library, but highlights the 118 hits identified and shown by the statistical values obtained from each test. Bubble size, colour, Z-score and t-test indicate statistical significance. The p-values within our cut-off of 0.01 represent 30% of the total hits while there are much less hits identified within the other cut-offs; Z-score = 4%, CV = 4.4% and SSMD = 15%. This may be explained by fact that the p-value represents the probability of seeing a value bigger than is observed, while the Z-score measures the performance in terms of the number of standard deviations away from the mean. The percentage cell viability statistic quantitates biological activity which may lead to the lower detection rates, while the SSMD directly measures the size of siRNA effect as represented by the strength of difference between a siRNA and a negative control. We found that the robust Z-score represented the reduction in cell viability better than the

SSMD, which may be due to the fact that SSMD is affected by variability between replicates. For example, the siRNA with the highest SSMD value of 145 from our screen, MECP2, had a mean and standard deviation of 0.4457 ± 0.017 , while IL5RA with a SSMD of 1.04 has a mean and standard deviation of 0.6817 ± 0.097 . This indicates that the SSMD is highly sensitive to the variation between the replicates, as opposed to necessarily representing the siRNA effect overall.

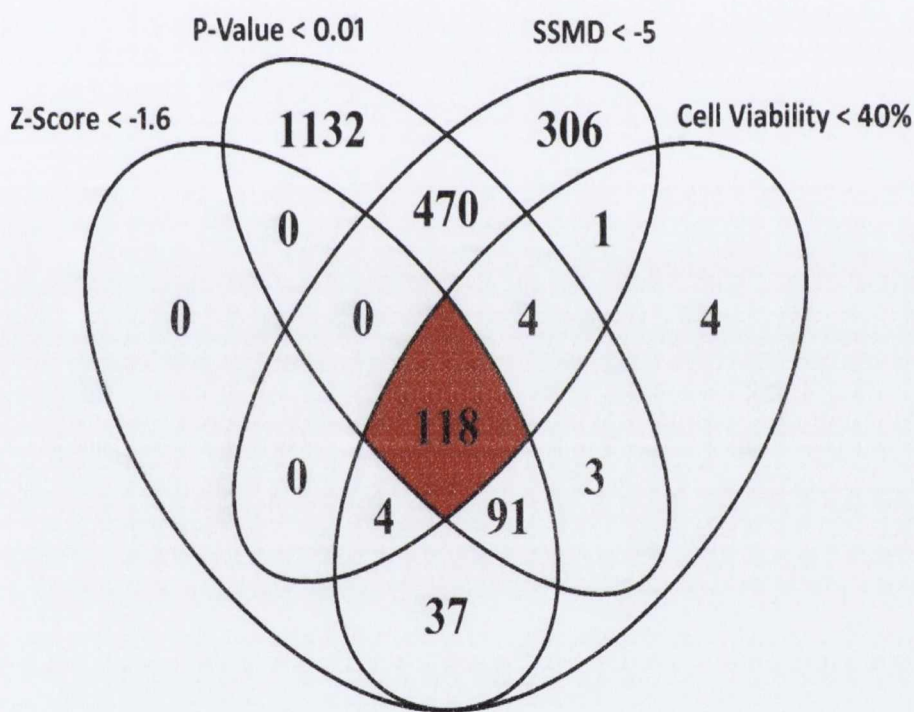


Figure 4.7: Venn Diagram Depicting Stringent Cut-offs for Each Statistical Test. Cut-off values were set as; p-value < 0.01, Z-score < -1.6, SSMD < -5, %CV < 40%. Results indicate 118 high confidence hits, as highlighted by a green arrow. These siRNAs were found within all 4 cut-offs, therefore were termed as high confidence.

Table 4.1: High Confidence Hits Ranked by Z-Score

Gene Name	Accession Number	Median Raw Values	Median Normalised Values	Median Z-Score Values	P Value	SSMD	Cell Viability	100 - CV
UBB	7314	0.067	0.107717	-3.03286	0.000348	-5.127522	10.7717	89.2283
RAN	5901	0.1	0.147275	-2.84195	0.000698	-6.875201	14.72754	85.27246
TPM1	7168	0.129	0.169514	-2.73463	0.002053	-8.355112	16.95138	83.04862
GTF2H4	2968	0.145	0.190789	-2.63195	0.000255	-14.59534	19.07895	80.92105
SYMPK	8189	0.139	0.219937	-2.49129	0.000206	-43.27717	21.99367	78.00633
SRPR	6734	0.19	0.222482	-2.479	0.001476	-30.10549	22.24824	77.75176
RAD51	5888	0.154	0.223837	-2.47247	0.000318	-23.22839	22.38372	77.61628
AOX1	316	0.146	0.22956	-2.44485	0.001392	-16.04781	22.95597	77.04403
CLASP1	23332	0.151	0.233746	-2.42464	0.000487	-5.548111	23.37461	76.62539
SH3KBP1	30011	0.16	0.23845	-2.40194	0.002843	-8.083709	23.84501	76.15499
KCNQ2	3785	0.164	0.240469	-2.3922	3.76E-05	-22.15481	24.04692	75.95308
SON	6651	0.154	0.243671	-2.37675	0.002948	-6.516021	24.36709	75.63291
RUNX2	860	0.125	0.246063	-2.3652	0.000699	-7.439641	24.6063	75.3937
TRPV2	51393	0.172	0.25425	-2.32569	0.00215	-13.14101	25.42498	74.57502
KRT10	3858	0.188	0.254398	-2.32498	0.008061	-6.273708	25.43978	74.56022
TREM2	54209	0.177	0.261641	-2.29003	0.001406	-15.90088	26.16408	73.83592
TGFB1	7040	0.224	0.262295	-2.28687	0.004818	-8.258522	26.22951	73.77049
TP53	7157	0.163	0.262903	-2.28393	8.22E-05	-13.14568	26.29032	73.70968
SDHD	6392	0.162	0.269551	-2.25185	0.00221	-6.48383	26.95507	73.04493
KCNAB1	7881	0.177	0.273148	-2.23449	0.002802	-7.018643	27.31481	72.68519
SLC14A1	6563	0.222	0.276119	-2.22015	0.001306	-35.22951	27.61194	72.38806
CD2BP2	10421	0.209	0.276455	-2.21853	0.000183	-5.309111	27.6455	72.3545
LPL	4023	0.18	0.283019	-2.18685	0.000111	-8.288104	28.30189	71.69811
CIRBP	1153	0.183	0.283282	-2.18559	0.000393	-6.739147	28.32817	71.67183
SYNJ1	8867	0.189	0.283358	-2.18522	0.002049	-6.788052	28.33583	71.66417
ISGF3G	10379	0.184	0.28417	-2.1813	0.000142	-16.16719	28.41699	71.58301
SVIL	6840	0.18	0.28481	-2.17821	0.001584	-9.297103	28.48101	71.51899
RABGEF1	27342	0.163	0.286972	-2.16778	0.004585	-5.280741	28.69718	71.30282
GPAA1	8733	0.224	0.294737	-2.1303	0.000515	-15.39577	29.47368	70.52632
HEY1	23462	0.162	0.297248	-2.11819	0.00233	-5.387744	29.72477	70.27523
DNAJA2	10294	0.242	0.297297	-2.11795	0.006006	-5.283339	29.72973	70.27027
TADA3L	10474	0.19	0.298742	-2.11097	0.000552	-10.193	29.87421	70.12579
BCL9	607	0.222	0.298789	-2.11075	0.007779	-10.45868	29.87887	70.12113
FOXG1C	2292	0.229	0.301316	-2.09855	0.00032	-32.76014	30.13158	69.86842
TBL1X	6907	0.172	0.301754	-2.09644	0.000718	-11.81628	30.17544	69.82456
PKP1	5317	0.231	0.303548	-2.08778	0.003503	-6.268723	30.3548	69.6452
CSEN	30818	0.215	0.303672	-2.08718	0.002811	-5.970275	30.36723	69.63277
UBA52	7311	0.225	0.304465	-2.08335	0.000477	-7.240774	30.44655	69.55345
TUBGCP3	10426	0.237	0.311432	-2.04973	0.000418	-23.99261	31.14323	68.85677
RELA	5970	0.266	0.311475	-2.04952	0.002196	-5.730606	31.14754	68.85246
CDC25C	995	0.2	0.312989	-2.04222	0.000396	-7.806416	31.2989	68.7011
USP30	84749	0.201	0.313084	-2.04176	0.000372	-6.59102	31.30841	68.69159

THBS2	7058	0.24	0.315375	-2.03071	0.002106	-5.521337	31.53745	68.46255
CORO2A	7464	0.206	0.318885	-2.01376	0.001213	-6.464036	31.88854	68.11146
NPTXR	23467	0.229	0.323446	-1.99175	0.000905	-7.489075	32.34463	67.65537
ABCC10	89845	0.216	0.324812	-1.98516	0.002269	-10.45389	32.4812	67.5188
GPC3	2719	0.247	0.325	-1.98425	0.000638	-9.103586	32.5	67.5
TF	7018	0.206	0.325949	-1.97967	0.0011	-9.115191	32.59494	67.40506
CYP2D6	1565	0.25	0.328084	-1.96937	0.000476	-5.953381	32.8084	67.1916
DUSP9	1852	0.212	0.331768	-1.95159	0.001863	-6.083605	33.17684	66.82316
LIF	3976	0.235	0.331921	-1.95085	0.002783	-6.941046	33.19209	66.80791
SIX6	4990	0.169	0.332677	-1.9472	0.002622	-9.045014	33.26772	66.73228
MYL7	58498	0.209	0.337097	-1.92588	0.003688	-5.950756	33.70968	66.29032
C8A	731	0.277	0.337805	-1.92246	0.00178	-13.66068	33.78049	66.21951
HIRIP3	8479	0.257	0.338158	-1.92075	0.001703	-6.947814	33.81579	66.18421
SIGLEC11	114132	0.214	0.338608	-1.91858	0.00045	-9.483855	33.86076	66.13924
PRKAB1	5564	0.234	0.33913	-1.91606	0.002663	-5.64749	33.91304	66.08696
TFAP2C	7022	0.216	0.339623	-1.91369	0.000457	-8.349282	33.96226	66.03774
SCYE1	9255	0.291	0.340749	-1.90825	0.006361	-8.15882	34.07494	65.92506
PNMT	5409	0.208	0.344942	-1.88801	0.001336	-7.093152	34.4942	65.5058
KIF2C	11004	0.221	0.345853	-1.88362	0.003598	-8.001195	34.58529	65.41471
APBB3	10307	0.23	0.345865	-1.88356	0.004227	-8.305002	34.58647	65.41353
DNTT	1791	0.268	0.351706	-1.85537	0.001229	-6.854378	35.1706	64.8294
RABL2B	11158	0.236	0.353823	-1.84515	0.006398	-10.51479	35.38231	64.61769
CPT2	1376	0.191	0.35568	-1.83619	0.00153	-23.89563	35.56797	64.43203
CASP10	843	0.203	0.35614	-1.83397	0.000839	-10.93635	35.61404	64.38596
NRCAM	4897	0.273	0.356397	-1.83273	0.003416	-106.6379	35.63969	64.36031
RAI1	10743	0.203	0.357394	-1.82792	0.007698	-9.09808	35.73944	64.26056
CLPP	8192	0.193	0.359404	-1.81822	0.001704	-23.65794	35.94041	64.05959
K- ALPHA-1	10376	0.229	0.360063	-1.81504	0.000375	-6.289968	36.00629	63.99371
ITGB3	3690	0.259	0.360223	-1.81427	0.001886	-5.365964	36.02225	63.97775
TG	7038	0.308	0.360656	-1.81218	0.001385	-31.01243	36.06557	63.93443
TRAF2	7186	0.251	0.364826	-1.79206	0.002343	-7.680998	36.48256	63.51744
HERC5	51191	0.274	0.365333	-1.78961	0.005548	-13.20927	36.53333	63.46667
MEP1B	4225	0.222	0.366337	-1.78476	0.00319	-5.076928	36.63366	63.36634
NFKBIL2	4796	0.208	0.368142	-1.77605	0.001791	-5.386924	36.81416	63.18584
ETF1	2107	0.281	0.369251	-1.7707	0.000615	-23.12719	36.9251	63.0749
CIQA	712	0.275	0.370121	-1.7665	3.42E-05	-5.938498	37.01211	62.98789
PEX13	5194	0.3	0.373134	-1.75196	0.004883	-8.787193	37.31343	62.68657
RDX	5962	0.284	0.373193	-1.75167	0.00031	-10.26999	37.31932	62.68068
SEC14L3	266629	0.283	0.373351	-1.75091	0.001696	-20.12735	37.33509	62.66491
PPM1E	22843	0.234	0.373802	-1.74874	0.002306	-22.81646	37.38019	62.61981
USP18	11274	0.241	0.375389	-1.74107	0.001292	-7.534152	37.53894	62.46106
MAD	4084	0.244	0.376834	-1.7341	0.001039	-7.750049	37.6834	62.3166
IFNW1	3467	0.261	0.377168	-1.73249	0.000213	-7.703439	37.71676	62.28324
GSTZ1	2954	0.26	0.382916	-1.70475	0.005664	-14.95241	38.29161	61.70839
PEPP2	54477	0.294	0.383812	-1.70043	0.002301	-8.138798	38.3812	61.6188
LAMR1	3921	0.272	0.384181	-1.69865	0.001382	-10.37466	38.41808	61.58192

ICAM1	3383	0.266	0.384393	-1.69762	0.001009	-5.781329	38.43931	61.56069
RRM2	6241	0.214	0.384892	-1.69522	0.003659	-6.612658	38.48921	61.51079
WNT8B	7479	0.261	0.385809	-1.69079	0.005291	-9.008995	38.58093	61.41907
IL9R	3581	0.278	0.386648	-1.68674	0.004014	-5.330966	38.66481	61.33519
CIB1	10519	0.248	0.388106	-1.6797	0.001049	-5.743023	38.81064	61.18936
LISCH7	51599	0.283	0.388736	-1.67666	0.003618	-5.675129	38.87363	61.12637
KIF1C	10749	0.249	0.389671	-1.67215	0.005401	-6.245861	38.96714	61.03286
TRIM28	10155	0.293	0.390667	-1.66735	0.000123	-5.428506	39.06667	60.93333
FBXL11	22992	0.293	0.390667	-1.66735	0.001492	-10.91748	39.06667	60.93333
ATF7IP	55729	0.26	0.390977	-1.66585	0.00653	-6.126194	39.09774	60.90226
CES1	1066	0.21	0.391061	-1.66544	0.000106	-11.15881	39.10615	60.89385
TLR2	7097	0.334	0.391101	-1.66525	0.003457	-18.99032	39.11007	60.88993
PROK1	84432	0.334	0.391101	-1.66525	0.007939	-7.373039	39.11007	60.88993
CAPN2	824	0.223	0.391228	-1.66464	0.001758	-7.446653	39.12281	60.87719
TAT	6898	0.261	0.391304	-1.66427	0.006117	-12.458	39.13043	60.86957
KCNJ8	3764	0.268	0.392962	-1.65627	0.00124	-11.0729	39.29619	60.70381
TNFRSF8	943	0.268	0.396157	-1.64085	0.003767	-13.27703	39.61567	60.38433
BAIAP1	9223	0.264	0.396992	-1.63682	0.002131	-15.84832	39.69925	60.30075
SRD5A1	6715	0.221	0.397482	-1.63446	0.002339	-5.844613	39.7482	60.2518
LDB1	8861	0.258	0.398456	-1.62976	0.00262	-12.18055	39.84556	60.15444
HOXC5	3222	0.259	0.4	-1.6223	0.000658	-14.27077	40	60
PTCRA	171558	0.234	0.4	-1.6223	0.001048	-6.246468	40	60
PSMD8	5714	0.235	0.401709	-1.61405	0.007838	-18.62681	40.17094	59.82906
SLC6A2	6530	0.323	0.401741	-1.6139	0.001197	-6.0061	40.17413	59.82587
TGFB2	7042	0.229	0.401754	-1.61384	0.000919	-11.44681	40.17544	59.82456
MNDA	4332	0.279	0.402597	-1.60977	4.94E-05	-6.678804	40.25974	59.74026
IFNA7	3444	0.244	0.40264	-1.60956	0.004079	-8.400837	40.26403	59.73597
CRISP1	167	0.332	0.403893	-1.60352	0.007283	-7.411065	40.38929	59.61071
MSH4	4438	0.28	0.40404	-1.60281	0.000859	-7.708515	40.40404	59.59596
GMPR2	51292	0.237	0.404437	-1.60089	0.007458	-7.296794	40.44369	59.55631

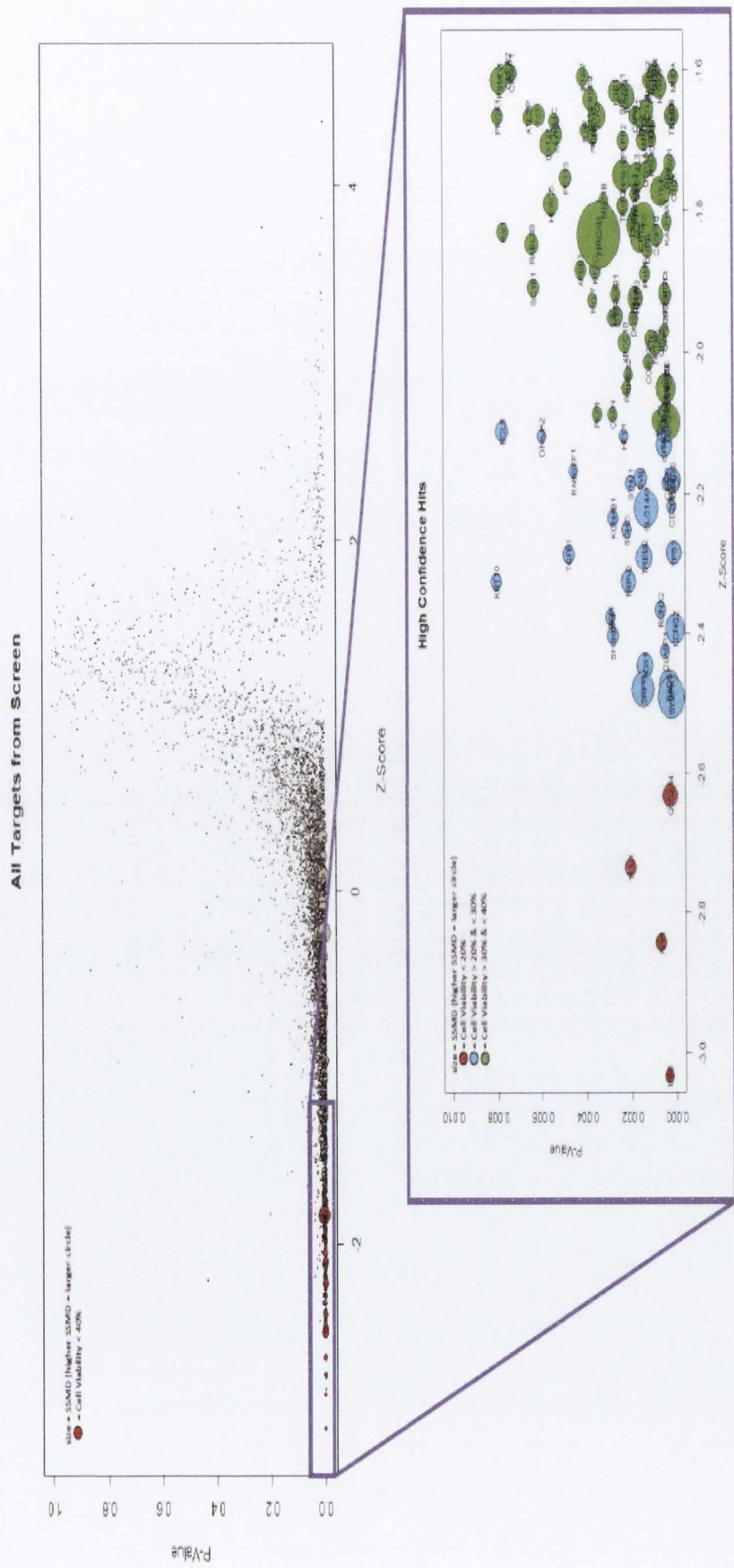


Figure 4.8: Bubble Graph of Hit Selection. This bubble graph, supplied by Dr.Fiona Behan, shows the top 118 high confidence hits sorted by all four statistical tests. Bubble size indicates SSMD value; colour indicates cell viability; x-axis represents Z-Score; y-axis indicates P-values

4.3.3.2.3 Confirmation of Knockdown of Known Cancer Promoting Genes

As supporting evidence that the siRNA screen was successful, a number of genes known to play key roles in cancer development and progression were identified within the top 20 hits (Table 4.1); including RAN, RAD51 and TP53. The Ras superfamily is known to play a key role in cellular physiology by regulating the nucleocytoplasmic transport of molecules through the nuclear pore complex (Sorokin et al., 2007). RAN has been identified as being overexpressed in several cancer types including pancreas, lung, ovarian and oesophageal, resulting in poor patient prognosis (Barres et al., 2010, Ning et al., 2014, Zhang et al., 2014, Deng et al., 2013). In relation to oesophageal cancer, one study investigated the role of RAN in relation to how miR-203 exerts tumour suppressor effects. It was shown that miR-203 is down-regulated in many tumour types including OSCC, and that ectopic expression can induce apoptosis along with reducing cell proliferation, migration and proliferation. With respect to RAN, it was shown that it is a direct target of miR-203 and that exogenous expression of RAN in miR-203-transfected cells, can reverse the tumour suppressive role of miR-203 (Zhang et al., 2014). RAD51 plays a key role in homologous repair and it has been shown that if defects exist in recombinant mediators such as BRCA1 or BRCA2, then RAD51 can be overexpressed. Given this, it is common to find RAD51 overexpressed in cancers, especially breast cancer, in which BRCA1 and BRCA2 mutations are common (Subramanyam et al., 2013). It has also been suggested that destabilisation of the genome by RAD51 may contribute to tumour progression (Richardson et al., 2004). RAD51 can also reduce NSCLC cell viability significantly ($p < 0.01$), demonstrated using three different cell lines; A549, H520 and H1703, which can be increased with gemcitabine treatment (Tsai et al., 2010). A link has also been identified between RAD51 and TP53 in which wild-type p53 protein can

suppress RAD51 transcriptional expression and protein activity (Buchhop et al., 1997). TP53 is a known tumour suppressor that plays an important role in modulation of cell proliferation. TP53 mutations are common in cancers, including OAC, with some contributing to over-expression of p53 protein as well as tumour differentiation and decreased post-operative survival rates (Madani et al., 2010). Therefore, silencing of TP53 by siRNA can reduce cancer cell proliferation if a mutation occurs in which p53 is over-expressed. This was indicated by siRNA mediated silencing of TP53 in bladder cancer cell lines 5637 and T24, which reduced cell viability by 58% and 60% respectively, at 72hrs post-transfection (Zhu et al., 2013b).

All three of these genes are over-expressed in cancer, and therefore it was expected that knockdown of these genes by siRNA would result in reduced cell viability as confirmed by previous studies (Barres et al., 2010, Zhu et al., 2013b, Tsai et al., 2010). From our screen it was shown that inhibition of RAN, RAD51 and TP53 resulted in a decrease in cell viability of 85%, 77% and 73% respectively (Table 4.1). These results indicating that the siRNA screen targeted genes that are over-expressed in OAC, and successfully inhibited their function. We can thus be confident that the novel hits identified act in the same manner; are over-expressed in OAC, and their inhibition significantly reduces cell viability.

4.3.4 Panther Classification Analysis of High Confidence Hits

To sub-categorise the top 118 hits by biological process, the Panther classification database was utilised. The list of 118 high confidence hits were input to the database and results indicated that 48.2% of the hits were associated with metabolic processes including proteolysis, protein modifications and translation. Genes involved in these

metabolic processes included UBB, C8A and CASP10. Ubiquitin B (UBB) is the top hit generated from our screen, and plays a key role in targeting cellular proteins for degradation by the 26S proteasome, maintenance of chromatin structure and regulation of gene expression. It has a known link to Alzheimer's disease (AD) (Hope et al., 2003) which was identified by investigating conditional expression of UBB+1 in human neuroblastoma cells which was shown to cause inhibition of the 26S proteasome. UBB+1 is a mRNA encoding variant of UBB which is the product of molecular misreading, most likely caused by transcriptional slippage of the UBB gene (Hope et al., 2003, van Leeuwen et al., 1998). The next largest category (43.9%) included cellular processes such as cell communication and cell cycle. Within this subset, 26 genes were identified as playing a role in cell communication including RELA, LIF and TP53. Both of these major biological processes are as expected, again confirming that the main hits found are involved in processes known to modulate cancer development.

The top 4 pathways identified by Panther were; gonadotropin releasing hormone receptor pathway which included TGF β -1 and -2, RELA, LDB1 and AMPK; apoptosis signalling pathway which included NF κ B, TP53 and Caspase 10; Wnt signalling pathway which included WNT8B and Bcl-9; and finally the p53 pathway which included TRAF2 and RRM2. Besides the first pathway, the three other pathways are all to be expected, as we are analysing effect on cell death, in which p53 and the Wnt pathway are both known to be highly involved. Identification of 5 hits within the gonadotropin releasing hormone receptor pathway was not initially as expected, but extensive literature reviews have identified its involvement in the tumourigenesis in many cell types including breast, ovarian and pancreas (Hackshaw,

2009, Kang et al., 2003, Grundker et al., 2011). Within the oesophageal setting for example, it has been demonstrated that RELA gene amplification and protein overexpression is significantly correlated with PLK1 expression in OSCC tissues (Lin et al., 2011). OSCC tissues were shown to have a significantly higher level of RELA (30-35%) which was undetectable in normal oesophageal tissue which was also shown to correlate with high PLK1 levels. RELA can also bind to and transcriptionally regulate the PLK1 promoter resulting in stabilisation of β -catenin protein.

4.3.5 Chromosomal Locations of Hits

GeneSpring was utilised to investigate the chromosomal locations of our hits. In this analysis, we utilised the extended list defined by Z-Score cut-off of -1.6, SSMD cut-off of -5 and which were also contained within a P-value <0.01 and %CV < 40%, which would be taken together and used for further analysis. This resulted in 1031 gene hits (Appendix 8.5). The next step was to determine if there was a high density of our hits located in any specific chromosomal locations.

Chromosome 17 contains 69 of the genes on our hit list of a total of 1531 chromosomal genes. Located on chromosome 17 are hits from our screen such as UBB which was our top hit and TP53 which is a well-known tumour suppressor. A total of 14 (23% of the total per chromosome 17) of our hits are located within the region 17q21, including KRT10, a gene whose product is known to modulate epithelial cell viability (Chen et al., 2006) and which is mainly associated with epidermolytic hyperkeratosis and ichthyosis with confetti (Arin et al., 1999, Burger et al., 2012). Recently KRT10 has been identified as a downstream molecule of PTEN

and demonstrated to improve cisplatin-resistance in ovarian cancer cell line C13K (Wu et al., 2014a). PTEN is a known tumour suppressor gene, and previous work by the same group has shown that it has the ability to reverse cisplatin-resistance in C13K cells. Within the oesophageal setting, a recent study has identified oral leukoplakia (Lee et al., 2000) cells as being immune-positive for KRT10 (Kobayashi et al., 2012). Also within the same region as KRT10, is BRCA1 which is known to be associated with hereditary breast cancer (Aberle et al., 1995). As is well known, BRCA1 is a tumour suppressor gene, but mutations within it can lead to development of breast cancer. For example, a recent study has identified the BRCA1 rs799917 CC genotype increases risk of OSCC compared to the TT genotype, in a Chinese case-control study (Zhang et al., 2013c). The region, 17q21 has also recently shown an association with epithelial ovarian cancer (EOC) following a GWAS study (Permut-Wey et al., 2013), which identified several genes which may confer risk of development of EOC.

Chromosome 1 contains 109 hits from our screen including BCL-9 and IL6R which are both located within 1q21. A total of 21 (19.26% of the total per chromosome 1) of our hits are located within this region (1q21) whose amplification is known to be involved in several cancer types including multiple myeloma and lung adenocarcinoma (Grzasko et al., 2013, Beroukheim et al., 2010, Weir et al., 2007). One study in particular detected that amplification of this region was found in 47.1% of multiple myeloma patients tested (Grzasko et al., 2013). This study demonstrated that following treatment with thalidomide, progression-free survival was 3 times higher ($p=0.002$) in patients without the amplification compared to those with the 1q21 amplification, while overall survival (OS) was similar, with a median OS of 26.6

months in patients with the amplification as compared to an OS of 62.4 months in patients negative for the amplification ($P=0.018$). This concluded that prognosis in myeloma patients with the amplification, is significantly worse than in myeloma patients without it.

Chromosome 6 also appears to be of particular interest, with 60% of the total hits per chromosome 6 being located at 6p21, a known immune related section which includes the major histocompatibility complex (MHC) region. This region has been highlighted as being of key importance in innate immunity and autoimmunity (Horton et al., 2004), and has been highlighted in a recent GWAS study of OSCC (Shen et al., 2014). This study highlighted 3 genes; HLA-DQA1, TRIM27 and DPCR1, as being located at key susceptibility loci, within a Chinese population, with the level of IHC staining of both HLA-DQA1 and TRIM27 being higher in OSCC samples than the neighbouring normal squamous oesophagus. Interestingly 6p21 has also recently been highlighted with regard to BO (Su et al., 2012), which identified a variant at locus rs9257809 corresponding to an area between two olfactory receptor genes *OR2D12* and *OR2D13* ($p=4.09 \times 10^{-9}$). Following correspondence with the investigators, (Su et al., 2012), none of the genes identified within our screen had been highlighted in the GWAS study they performed, but it is our belief that this region may still be of interest and hold potential as a possible target region for BO and OAC treatment. Given this, further investigation is currently being undertaken by other members of the team who are currently validating two of our hits, TREM2 and RUNX2 both of which are located at 6p21.1 and will be discussed further throughout this thesis.

Chromosome 12 contained 50 hits from our screen, representing 3.9% of the total gene on that chromosome, including UBC and RAN. Both of these genes are located at 12q24, with a total of 15 (30% of the total per chromosome 12) of our hits being located here. Genetic variations at 12q24 have been implicated in a number of cancers including breast and colorectal cancers (Qin et al., 2013b, Cicek et al., 2012, Ghousaini et al., 2012). A genome wide study has also highlighted a genomic variation at 12q24 associated with OSCC, although neither of the genes highlighted were contained within our top 1031 genes as they did not reduce cell viability more than 60% (Cui et al., 2009). Due to the time constraints, further analysis of specific regions within the chromosomes themselves was not carried forward but it may prove of interest for further research.

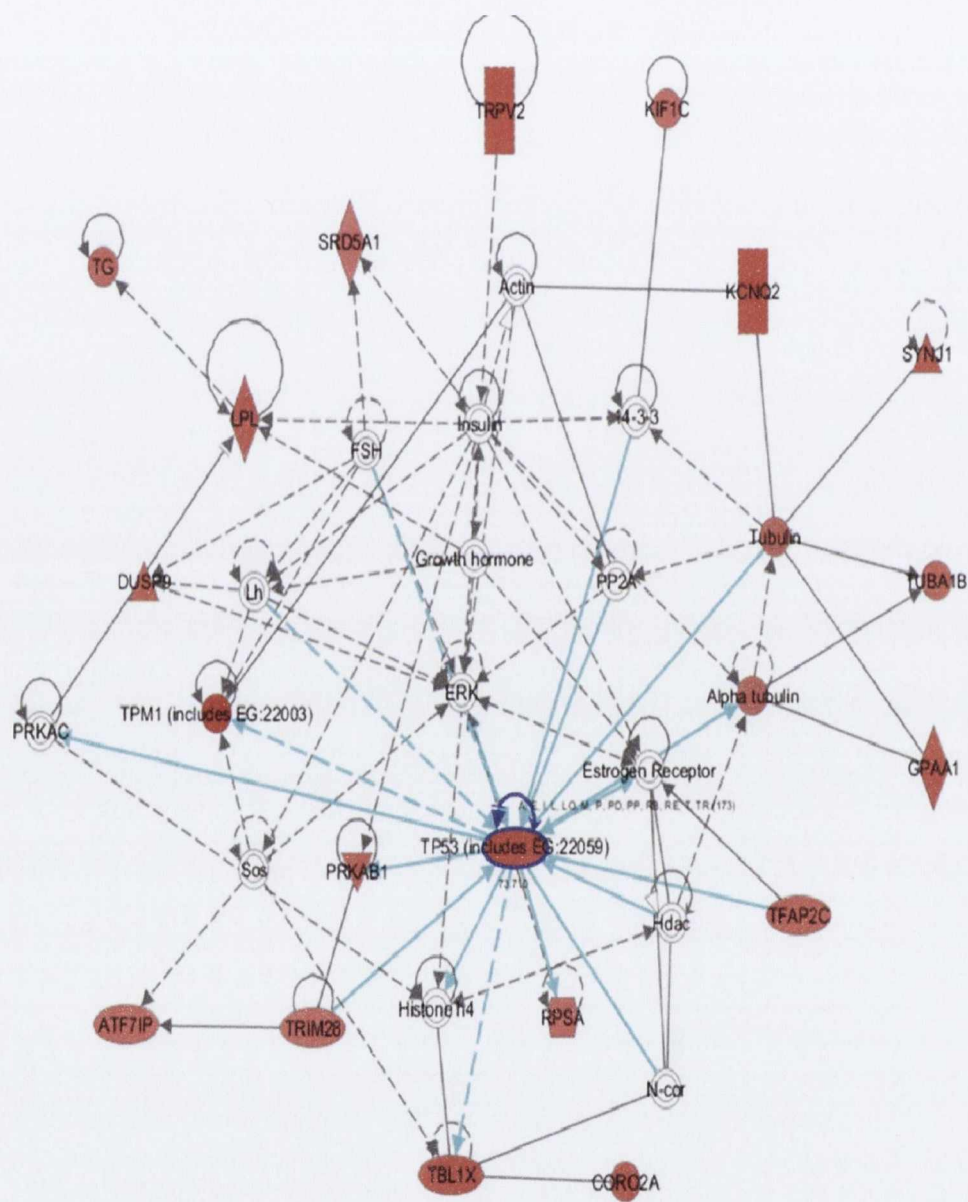
4.3.6 Informatics Results for High Confidence Hits

Following from the statistical and chromosomal locations analyses, bio-informatics analysis was performed using IPA (Figure 4.9) and DAVID gene ontology software (Figure 4.10). Bio-informatical analysis is used after identification of 'hits' to elucidate their putative mechanism(s) of action. There are many public and private websites that can be utilised for this purpose that contain large dataset results from microarrays, RNAi screens and proteomic studies. For our bioinformatics analysis we utilised two specific packages, each giving a different output; gene ontology and pathway analysis. The top 1031 genes identified as hits following statistical analysis were input in each of these packages; DAVID and IPA.

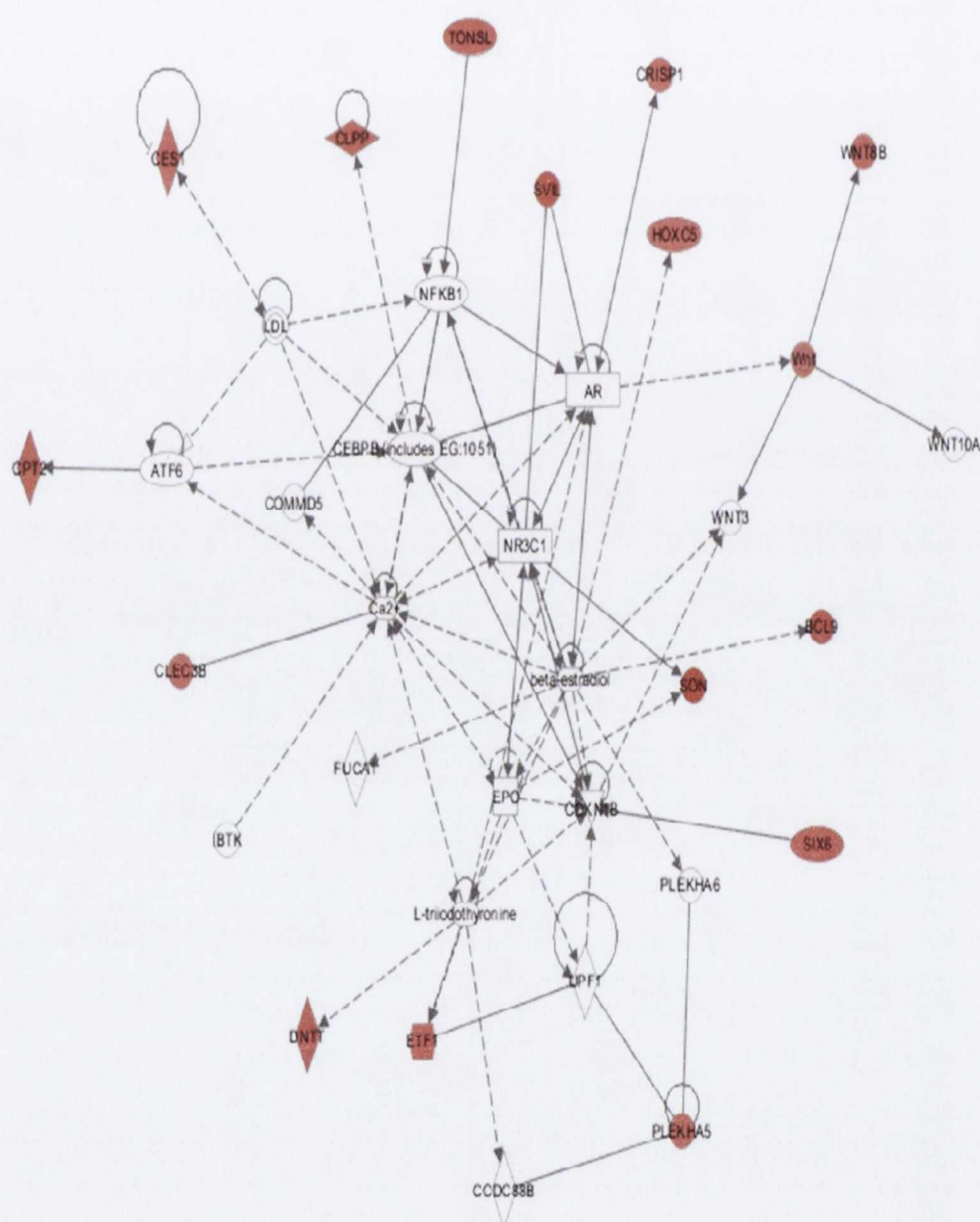
4.3.6.1 Ingenuity Pathway Analysis (IPA)

Gene pathway analysis utilises all published works and creates maps of known interacting genes to identify gene functions, canonical pathways, transcription factors and networks. Ingenuity Pathway Analysis (IPA) is a database that utilises this format to elucidate gene interactions and pathways, thus the top 1031 siRNAs generated following the statistical analysis of our data was input into the IPA database. It was also run using all hits within the 118 high confidence hit cut-offs (Z-Score of -1.6, P-Value of 0.01, SSMD of -5 and CV of 40%). Figure 4.9 highlights the top 4 networks and the general functions these '*high confidence hits*' genes are involved in. Genes highlighted in red are the genes/data generated from our screen, while outlined in blue are the main interactors within the networks such as TP53, NFκB and TGFβ, which are also identified as hits. General functions indicated areas such as haematopoiesis and haematological system development, along with expected functions such as cellular development and cellular growth and viability.

Network 1: Small Molecular Biochemistry, Endocrine System Development and Function, Drug Metabolism



Network 3: Antigen Presentation, Cellular Growth and Proliferation, Haematological System Development and Function



Network 4: Cellular Movement, Connective Tissue Development and Function, Cell-To-Cell Signalling and Interaction

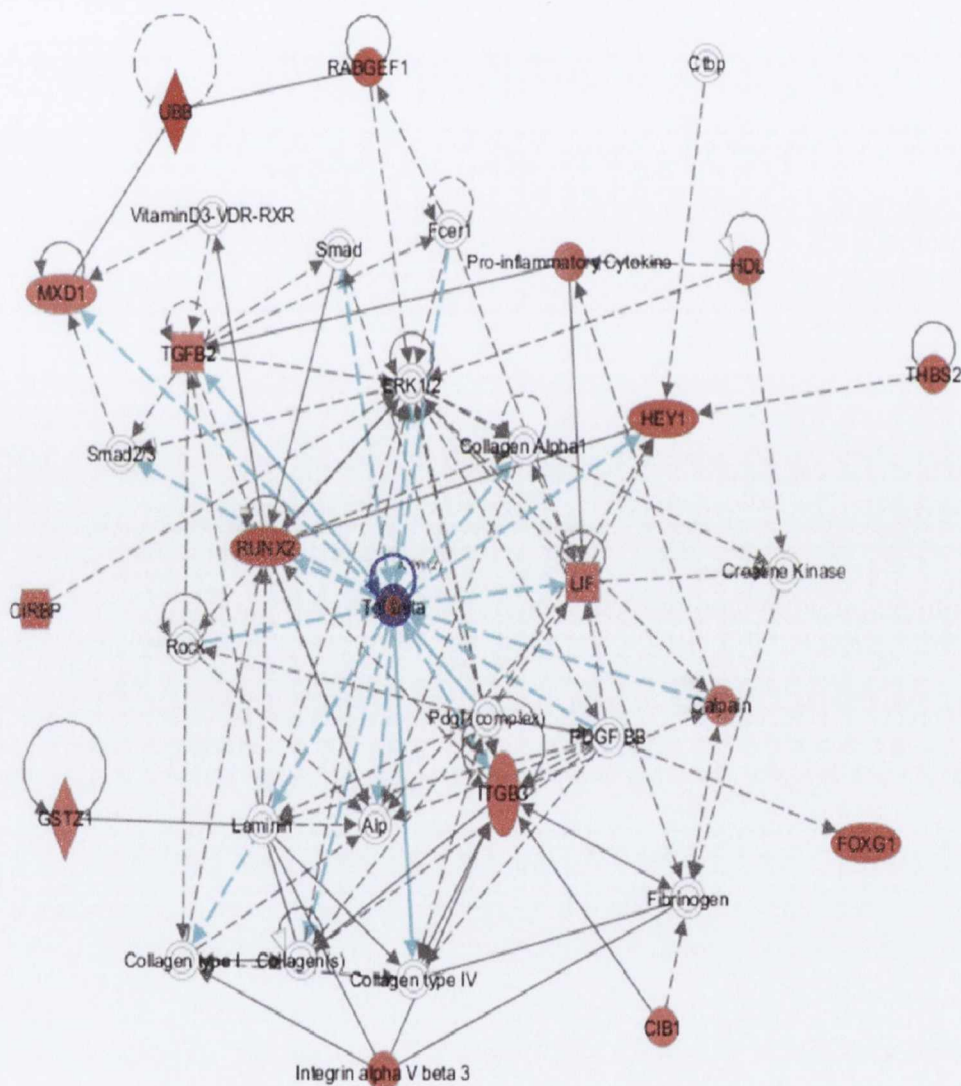


Figure 4.9: IPA Pathways Indicating the top 4 Network Associations for our 118 High Confidence Hits (HCH). Cut-off ranges used were Z-Score<-1.6, SSMD<-5, P-Value<0.01 and CV<40%.

4.3.6.2 DAVID Gene Ontology

DAVID is a web-based programme that focuses on gene ontology classifications. It acts as a data mining tool that is capable of systematically combining functionally descriptive data with intuitive graphical displays (Dennis et al., 2003). Simply, the user can take a gene and generate a list of biological functions associated with it, therefore gaining further knowledge about differing diseases that it is associated with. It also groups together several genes from a list according to several gene ontology classifications such as biological process, molecular function and cellular component.

The 1031 genes that were used for IPA analysis were also run through DAVID gene ontology software which provides functional annotation for the investigation of the biological meaning of a large list of genes. Results identified 57 clusters highlighting biological areas such as involvement in cell signalling, cell cycle and cell development. All of which are to be expected when analysing effect on cell viability. From Figure 4.10A, we can see that the second cluster identified an association with immune function, including immune and inflammatory responses. Further study of this cluster identified genes from our list (Figure 4.10B) that were included within this cohort, including leukaemia inhibitory factor (LIF) and complement component 1q subcomponent a chain (C1QA) which are highlighted in red boxes. Inflammation and the immune response have thus been highlighted as an area of interest resulting from our screen. Given this, genes highlighted within this area were chosen for verification along with others with known involvement in cancer e.g. RAD51 and SON. This verification step would serve to confirm knockdown in the GOhTRT cells line while further analysing the effect of knockdown in several oesophageal cell lines.

A

Annotation Cluster 2 Enrichment Score: 1.5026512356236937

Category	Term	Count	%	PValue	Genes	Fold				
						Log Total	Pop Hits	Pop Total	Enrichment	Benjamini
GOTERM_BP_FAT	positive regulation of immune system process	8	0.406713727	0.002561093		107	238	13526	4.249744758	0.38082842
GOTERM_BP_FAT	positive regulation of immune response	6	0.305033045	0.005527505		107	145	13526	5.23156234	0.999769335
GOTERM_BP_FAT	positive regulation of response to stimulus	7	0.355871886	0.010508341		107	236	13526	3.750039601	0.99999856
GOTERM_BP_FAT	defense response	11	0.55922725	0.02252365		107	615	13526	2.261347922	1.056734466
GOTERM_BP_FAT	response to wounding	10	0.508389409	0.023163485		107	530	13526	2.385469935	1.0563424773
GOTERM_BP_FAT	activation of immune response	4	0.203355363	0.037522021		107	94	13526	5.379996023	1.059963987
GOTERM_BP_FAT	inflammatory response	7	0.355871886	0.042464607		107	325	13526	2.723105679	1.0634381644
SP_FIR_KEYWORDS	innate immunity	3	0.152515523	0.071232357		109	78	19235	6.787226535	0.999999999
GOTERM_BP_FAT	immune response	10	0.508389409	0.091539544		107	690	13526	1.832317486	1.0722855852
GOTERM_BP_FAT	innate immune response	4	0.203355363	0.094166597		107	138	13526	3.664634972	1.0724101115
SP_FIR_KEYWORDS	immune response	3	0.152515523	0.358909087		109	224	19235	2.36340924	1.0917979414

B

Gene	innate				activation of		positive regulation of		positive regulation of		innate		positive regulation of		defense		response to	
	innate	innate	innate	innate	innate	innate	innate	innate	innate	innate	innate	innate	innate	innate	innate	innate	innate	innate
tumor protein p53	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
triggering receptor expressed on myeloid cells 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
leukemia inhibitory factor (chondrogenic differentiation factor)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
myeloid cell nuclear differentiation antigen	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
interferon, omega 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
interferon, alpha 7	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
tropomyosin 1 (alpha)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
intercellular adhesion molecule 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
potassium inwardly-rectifying channel, subfamily J, member 8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
TNF receptor-associated factor 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
aldehyde oxidase 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
transformin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
transforming growth factor, beta 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
transforming growth factor, beta 1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
v-rel reticuloendotheliosis viral oncogene homolog A (avian)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
tol-like receptor 2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
complement component 1, q subcomponent A chain	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
complement component 8, alpha polypeptide	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1

Figure 4.10: DAVID Gene Ontology Results. A) Cluster 2 indicates a high enrichment score associated with immune processes; B) Detailed analysis of cluster 2 shows genes involved in immune response. Highlighted are two hits chosen for further verification and validation. (Figure 4.10 supplied by Dr. Shane Duggan).

The overall conclusions that can be drawn from the bio-informatic analysis of gene pathways and ontology are; that pathways involved in cell cycle, cell death and cellular growth were identified as expected when analysing treatment effect on cell viability in relation to carcinogenesis. More interestingly, areas including haematopoiesis and the immune response were also highlighted warranting further investigation in relation to OAC. We have identified a large number of immune related genes that have been identified as hits as generated by our screen, that regulate GOhTRT cell proliferation. These genes will be carried forward for subsequent verification to confirm specificity of the siRNA, within the same cell line, before further verification in several cell lines.

4.3.7 Verification of Hits

Following bio-informatic analysis, a selection of 26 hits (Figure 4.11), were chosen for verification within the GOhTRT cells line. These genes were chosen as they were contained within our high confidence hits, and following an extensive literature search appeared biologically interesting in relation to immunity and inflammation. A cut-off of 50% cell viability was set indicating which siRNA targets would inhibit cell viability most significantly with results showing that 15 genes were under this threshold. Out of these 15 genes, 10 were carried forward for further verification, along with two other genes that we considered biologically interesting, TLR2 and SON. Toll-like receptors (TLR) have been identified in the pathogenesis of a number of cancers including gastric cancer (Kennedy et al., 2013) while SON has been identified as having sequence similarities to the MYC family of oncoproteins and is highly expressed in OSCC cells, indicating a possible role in cancer (Furukawa et al., 2012, Ahn et al., 2011). The five genes that were not carried forward are NR1H2, F8,

C8A, RAN and KCNQ2. NR1H2 is a liver X receptor that regulates macrophage function and is involved in lipid metabolism. C8A is involved in the formation of the membrane attack complex (MAC) in the complement pathway and its deficiency is associated with neisserial infections. F8 is a blood coagulation factor mainly linked to haemophilia while RAN is already widely studied and therefore was deemed unnecessary to verify further. KCNQ2 is a potassium channel and is mainly associated with neonatal seizures and epileptic encephalopathy. These genes were not selected as following an extensive literature search we considered them less biologically relevant than the other 12 in relation to immune factors in carcinogenesis. Therefore the 12 genes carried forward for verification were; C1QA, LIF, SYMPK, TRPC7, RUNX2, RAD51, KRT10, TRPV2, MND1, MXD1, SON and TLR2.

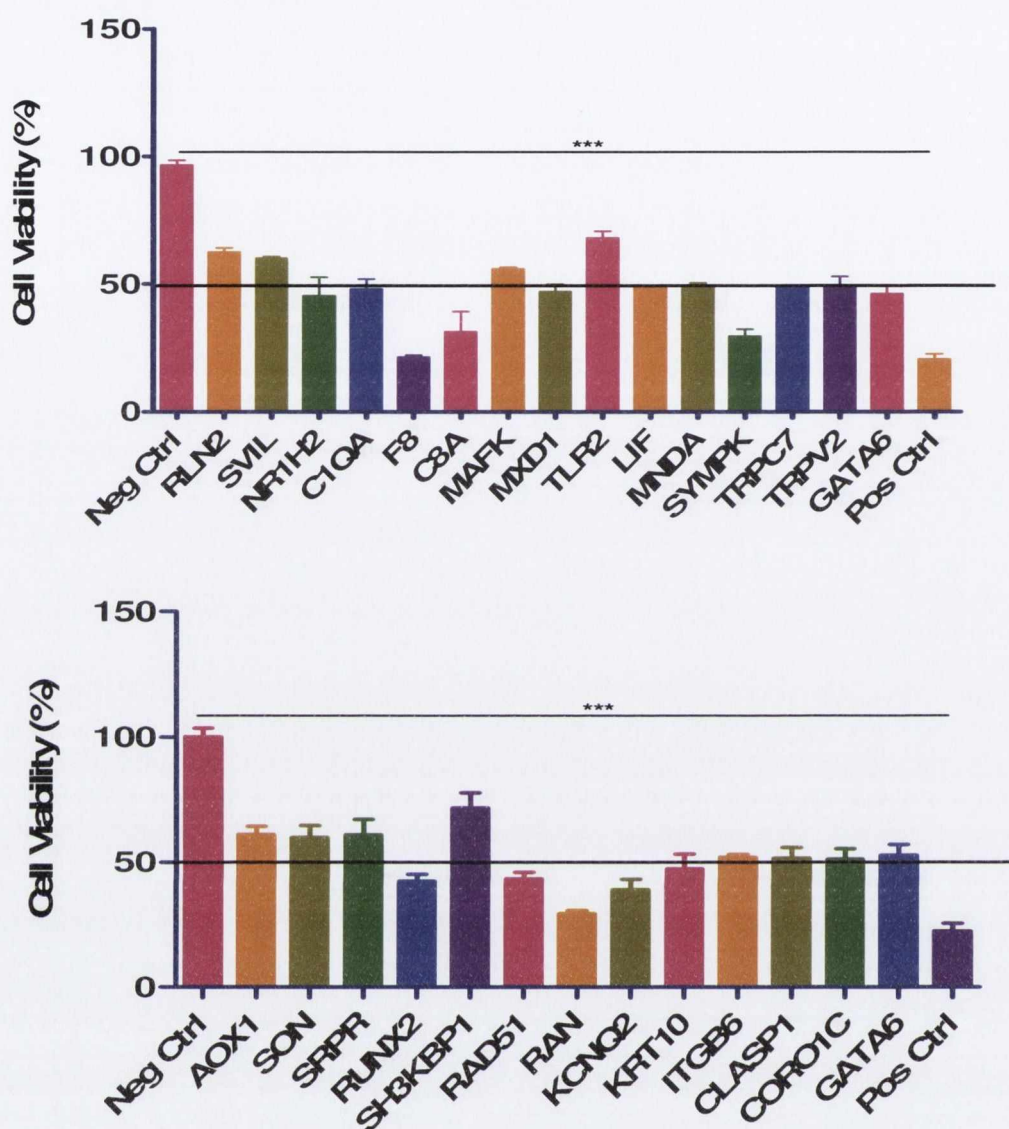


Figure 4.11: Verification Panel of 26 Genes Chosen Following Statistical and Bio-informatic Analyses. A selection of 26 genes, identified following statistical and bioinformatics analyses were verified via MTT assay. A cut-off value of 50% cell viability was used to identify genes that would be verified further in several cell lines. siRNA transfection conditions are as per table 2.1. N=3 and results are shown as mean $\pm$ SEM (t-test; ***p<0.0001)

Further verification of these 12 hits was undertaken in three cell lines including the GOhTRT, SK-GT-4 and EPC2-hTERT cell lines, which span the oesophageal

carcinogenic sequence (Figure 4.12). The SK-GT-4 cells line represents OAC, the GOhTRT cell line represents HGD and the EPC2-hTERT cell line was used as a model of “normal” oesophageal cells. Verification was undertaken to; firstly confirm knockdown in the GOhTRT cells line shown in Figure 4.11, then to analyse the effect of gene knockdown in the other two cell lines. The aim of this body of work is to identify a siRNA target that will reduce cancer cell viability; thus a reduction in cell viability in the SK-GT-4 and GOhTRT cell lines. If this was noted, then the effect of the siRNAs would be analysed in the “normal” cell line, EPC2-hTERT. It is hoped that inhibition of a specific gene(s) would result in a reduction in OAC and HGD cell viability, but would not affect EPC2-hTERT cell viability. As can be seen, this was not achieved, which may be due to the fact that the EPC2-hTERT cell line is transformed. The EPC2-hTERT cell line is a hTERT-transformed primary human oesophageal keratinocyte cell line, derived from a 55-year old male which are grown in Keratinocyte-SFM medium containing bovine pituitary extract and epidermal growth factor. The cells were taken from a morphologically normal proximal oesophagus region as determined by H&E staining indicating normal mucosa with no inflammation, dysplasia or cancer. They have a diploid karyotype (Andl et al., 2003), and are known to express E-cadherin as well as cytokeratins K4, K5, K13, and K14 (Kim et al., 2004). In general, effects in cell lines vary and they may not entirely represent the original tissue from which they were derived due to continual culture which may cause mutations and other changes. This issue has recently been highlighted in breast cancer cell lines when compared to breast cancer biopsy samples (Keller et al., 2010), and in particular the cell lines representing normal breast tissue differed dramatically, suggesting that adherent culture of normal cells may preferentially select for a particular subtype.

In Figure 4.12, it appears that some siRNAs selectively affect the SKGT4 OAC cell line as compared to the GOhTRT Barrett's oesophagus cell line. For example, inhibition of RUNX2 reduces GOhTRT cell viability by 77%, but only reduces SKGT4 cell viability by 15%, while inhibition of TRPV2 inhibits GOhTRT cell viability by 65% but reduces SKGT4 cell viability by 28%. This is an interesting finding and will be discussed further in section 4.4.

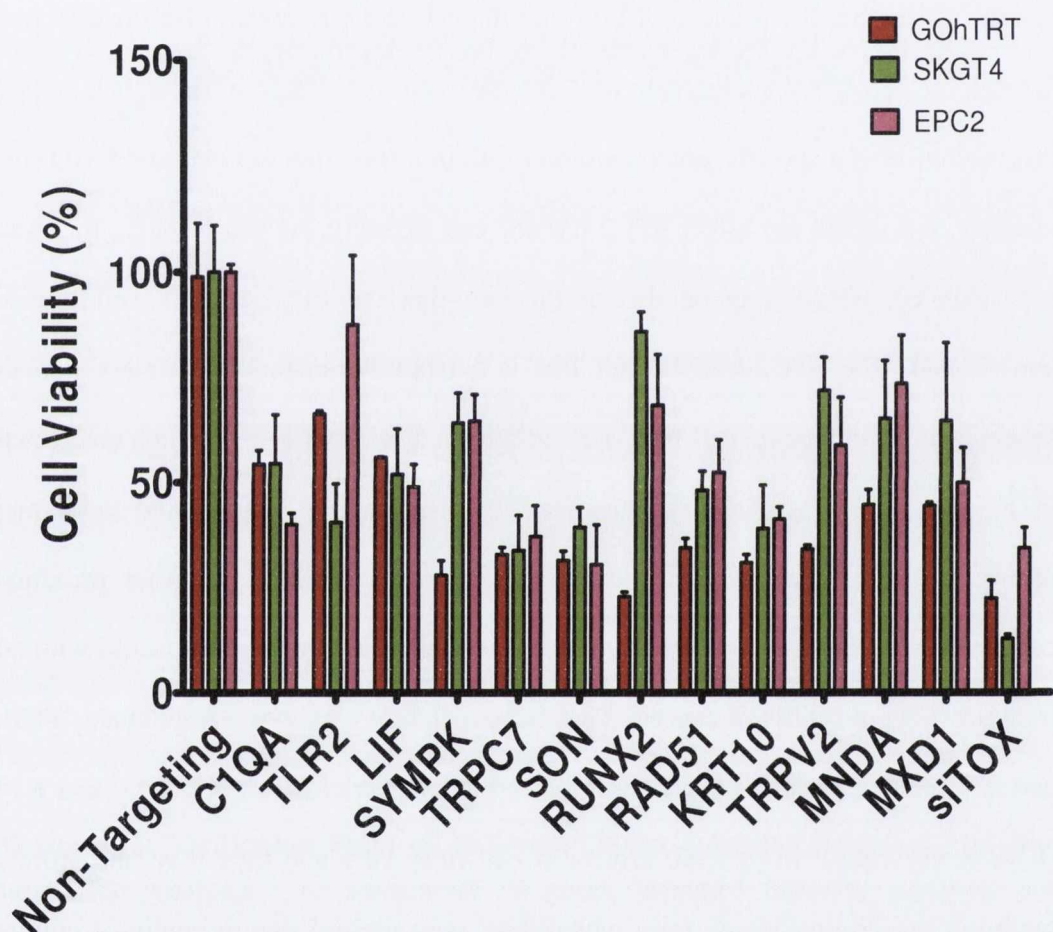


Figure 4.12: Verification of Hits in Three Cell Lines Spanning the Oesophageal Carcinogenic Sequence. Verification was undertaken in GOhTRT, SK-GT-4 and EPC-2 cells lines. siRNA transfection conditions for each cell line are as described in table 2.1. Cell viability was assessed by MTT assay. N=3 for each cell line. Results indicate mean $\pm$ SEM.

Observations indicate that treatment with all of the selected siRNA affect cell viability in all cell oesophageal cell lines. This indicates that the genes that are targeted may be necessary for oesophageal cellular proliferation. Further investigations in primary normal oesophageal cells are ultimately necessary to correlate with non-transformed cell growth. Three genes were identified whose treatments with specific siRNA reduced cell viability significantly in all cell lines, C1QA, LIF and TRPC7. These genes were then analysed using Oncomine database (Rhodes et al., 2004); a cancer microarray database and web-based data-mining platform that allows for determination of gene expression across several studies in numerous cancer types. Using this database it was shown that C1QA expression was 2.5 fold higher in OAC as compared to normal oesophagus in a study by (Kim et al., 2010) and further shows an increase in expression in a study by (Kimchi et al., 2005).. LIF was analysed the same way and expression appeared to be increased in BO and OAC as compared to normal oesophageal in both the Kim et al study and the Kimchi et al study, which is significant between normal and OAC. When TRPC7 was analysed in the same way it was noted that it is not up-regulated in either BO or OAC as compared to normal oesophageal tissue. It was for this reason that it was considered more biologically interesting to functionally validate C1QA and LIF only, which were also chosen as they are both secreted and therefore would be easier to target with drugs.

4.3.8 Sequence Analysis of LIF and C1QA Sequences

Basic Local Alignment Search Tool (BLAST) is used to identify similar regions between sequences. Thermo Scientific supplied sequences for the 4 pooled siRNAs contained within the siGENOME SMARTpool, for both LIF and C1QA. Each sequence was analysed using BLAST, and each was determined to be a 100% match to the gene target in humans. Another sequence analysis tool, Ensembl, can be used to determine specific isoforms of a protein. Leukaemia inhibitory factor (LIF) has three identified isoforms; LIF-D, LIF-M and LIF-T. LIF-D has been identified as biologically secreted, while LIF-M and LIF-T are both located intracellularly. Exons 2 and 3 are common to all isoforms, but they differ by having alternate first exons resulting in the 3 isoforms (Hisaka et al., 2004). Ensembl analysis of the sequences provided by Thermo Scientific for the siGENOME SMARTpool human LIF indicates that the LIF siRNA used was that of isoform LIF-D. This was concluded as one of the sequences provided is located within exon 1 of the LIF-D isoform which is highlighted in yellow in appendix tables 8.1 & 8.2. None of the sequences are present within exon 1 of either LIF-M or LIF-T but, of the four sequences located within the SMARTpool, at least one sequence is located within each of the 3 exons of LIF-D. Complement Component 1, Q Subcomponent, A Chain (C1QA) has 3 transcripts; C1QA_001, C1QA_002 and C1QA_003. The sequences supplied by Thermo Scientific for the C1QA siRNA were found to be located in 2 of the transcripts, C1QA_001 and C1QA_003. The second transcript C1QA_002 contains 3 exons, the same as the other 2 transcripts, but exon 1 in transcript 2 is an alternative 5' UTR (untranslated region). From these analyses we were able to confirm specificity of the siRNAs used were correct and unique to our genes of interest. Ensembl analysis using the supplied siRNA sequences also allowed for confirmation that all sequences

supplied within the SMARTpool were less than 30bp's. This detail minimises the risk of any innate immune response that may be triggered which may induce non-sequence specific down-regulation of protein expression (Pan et al., 2012).

4.4 Discussion

Statistical analysis was undertaken using four tests designed specifically for RNAi screen data analysis with stringent cut-offs resulting in the identification of 118 high confidence hits (Table 4.1), which was followed by bio-informatical analysis. By inputting our larger dataset (appendix 8.5) to the DAVID database we were able to identify several biological processes with which many of our genes were associated including secreted proteins within the extracellular region, innate immune response and cell cycle, which were the top 3 clusters gained from our data. The larger dataset was used to give a better perspective on a broad range of biological processes with which our genes may be involved in. Interestingly, 4 out of the top 10 clusters identified were associated with the immune system and inflammation. The second cluster (Figure 4.10A) identified that many of our hits played a role in the immune system, immune response and innate immunity.

The immune response is activated upon detection of infection or non-self molecules, and activated leukocytes have been found in high abundance within different tumour types. Leukocytes are derived from hematopoietic cells and there are 5 main types; macrophages, monocytes, basophils, eosinophils and lymphocytes. These immune cells communicate with each other by releasing secreted cytokines, which act to modulate the immune response. Activation of such genes results in triggering of the inflammatory response by release of cytokines such as IL-8 and IL-6 respectively,

which can result in reduced apoptosis, increased cell proliferation and angiogenesis. Both have been identified as playing a key role in inflammatory-induced carcinogenesis (Pikarsky et al., 2004, Zhang et al., 2013a) by potentially either promoting or inhibiting tumour progression by activating downstream transcription factors including for example NF κ B and STAT3.

Along with DAVID, IPA pathway analysis identified several gene networks, the top 4 of which are shown in Figure 4.9. Two of these networks have well known central regulatory genes, such as NF- κ B and TGF β which are known to regulate DNA transcription and cell viability respectively. Network 4, which has TGF β as its central regulator shows a direct effect on LIF. Following from statistical and bioinformatical analyses, the next step was to take a subset of these 118 high confidence hits, and verify them via MTT assay to confirm the screen results. As previously stated, the top 26 of the 118 high confidence hits identified were taken for verification in the GOhTRT cell line (Figure 4.11). The aim of this project was to identify genes that regulate OAC cell viability, which were previously not known to be involved in OAC, thus TP53 and ICAM1 were removed from the verification process as they already have a well-documented link to cancer development. ICAM1 activation is induced by the presence of pro-inflammatory cytokines including IL-1 and TNF, and a recent study has shown that ICAM1 levels are five times higher in BO patient samples as compared to controls ($p < 0.05$) (Alasehirli et al., 2014). The role of mutations within the TP53 tumour suppressor gene in OAC has been widely studied and it has been identified that patients with LOH of TP53 are 16-times more likely to progress to OAC (Reid et al., 2001). A recent study has also shown that in a cohort of 142 patients, 47% of the tumours from these patients demonstrated a TP53 mutation

(Madani et al., 2010) indicating the major role TP53 mutations play in cancer development.

Cellular transformations are necessary to overcome the finite life-span associated with the use of cell lines, which can be limited due to the progressive shortening of telomeres, but immortalisation is also a necessary step toward malignant transformation of normal human somatic cells. The EPC-2 cell line was derived from human oesophageal epithelial cells in primary culture, which were immortalised by telomerase, resulting in creation of the EPC2-hTERT cell line (Harada et al., 2003). Expression of the catalytic subunit of telomerase (hTERT) was used to immortalise the EPC2 normal cell line, which has a diploid status. Telomeric DNA erosion is a key factor in senescence, which can be antagonised during cell immortalisation and transformation. In the normal human oesophageal epithelial EPC2 cells, the hTERT expression was restored to normal therefore the EPC2-hTERT cell line was immortalised and showed wild-type p16 and p53 loci (Harada et al., 2003). Although the EPC-2 cells are hTERT transformed, in many studies it is used as a primary oesophageal epithelial cell line.

One of the limitations of this screen is the fact that it was performed in the high grade dysplastic cell line, GOhTRT. This cell line was used because it had previously been shown in the lab that it was resistant to DCA induced reduction in cell proliferation, and was thus considered of particular interest in terms of OAC developed from dysplasia caused by acid reflux. OAC cell lines were not used as they did not show consistent results when analysing effect on cell viability using the MTT assay. Other assays, including the AlamarBlue assay, were tested to see if consistency was

achieved, but this was not the found. As consistency and reproducibility were of key importance for reliable and significant results, the use of the GOhTRT cell line was chosen.

One of the main limitations of the use of HGD instead of OAC cells, is the that only 20-40% of patients with HGD progress to OAC (Collard, 2002, Rastogi et al., 2008, Reid et al., 2000) and also that the genetic conformation differs between HGD and OAC. For example, mutations within the tumour suppressor gene P53 are sometimes found in metaplastic non-dysplastic mucosa and in LGD, but the frequency of the mutations has been shown to be significantly increased in HGD and OAC, reaching 80% of cases in some series, with an even higher frequency of loss of heterozygosity at the p53 locus (Fléjou, 2005). Strong p53 protein expression has been identified in both HGD and superficial OAC, as compared to LGD and non-dysplastic metaplastic mucosa, using immunohistochemical (IHC) staining (Chatelain and Flejou, 2003), which has also been shown on other studies including one study that again looked at IHC staining of p53. In this study, positive p53 staining increased steadily for patients negative for dysplasia (0%), LGD (9%), HGD (55%) and OAC (87%) (Younes et al., 1993). Chromosomal deletions, amplification of oncogenes and growth factors may also play a key role in the promotion of neoplastic progression, several of which have been detected in OAC tissue samples and neighbouring mucosa utilising several different methods (Miller et al., 2003, Menke-Pluymers et al., 1996, Walch et al., 2000). Among the genomic amplifications suggested to play a role in OAC progression, in these studies, are c-myc, EGFR and the amplification of locus 20q13.2. A more recent study has shown that, in reference to these loci, amplification of at least one was observed in 14% of HGD and increased to 50% in OAC ($p=0.015$),

and the most frequently amplified locus was c-myc (18%), followed by 20q13 (13%) and EGFR (11%), while increased amplification levels of the loci were more frequent in OAC (72%) compared with HGD (20%; $p=0.049$) (Rygiel et al., 2008). Although these differences between HGD and OAC are important to highlight, the use of HGD cells is still significant, as genes identified using this cell line could be markers of the change from HGD to OAC. To determine if the genes identified are stage specific, they would need to be verified and validated in cells representative of normal oesophageal, LGD and OAC, as well as HGD.

The identification of a large proportion of immune related genes being involved in OAC cell viability in this body of work has given more weight to the belief that inflammation caused by acid reflux increases risk of progression to OAC. Gastroesophageal reflux disease can cause inflammation of the oesophageal epithelium leading to BO and an increased risk of developing OAC. The twelve genes indicated in Figure 4.12 are known immune related genes; C1QA, TLR2, LIF, SYMPK, TRPC7, SON, RUNX2, RAD51, KRT10, TRPV2, MNDA and MXD1. The role that each gene plays in the immune response has been highlighted in Table 4.2. As previously mentioned, Figure 4.12 suggests that inhibition of specific genes may have a selective effect on cell viability between the cell lines. For example, inhibition of RUNX2 shows a nearly fourfold decrease in cell viability between the GOhTRT cell line and the SKGT4 cell lines (22.7% vs 85.8%). This pattern was also noted following TRPV2 inhibition in which the reduction in cell viability seen in the SKGT4 cell line was doubled in the GOhTRT cell line (29% vs 64.8%), while inhibition of the toll-like receptor TLR2 in the SKGT4 cell line more than doubles the decrease seen in the GOhTRT cell line (60% vs 34%). The reason for this selectivity

could be due to the expression level of the genes in both BO and OAC. For example, one study analysed the effect of SOX2 inhibition in a number of lung and oesophageal cell lines. It was shown that inhibition of SOX2 by shRNA reduced proliferation in the four cell lines that were known to amplify the 3q26.33 region, in which SOX2 is located, but not in a control cell line which lacked significant SOX2 expression (Bass et al., 2009). Utilisation of Oncomine did not identify any significant changes in expression in either BO or OAC tissue samples for; RUNX2, TLR2 or TRPV2, when comparing expression in normal oesophageal, BO and OAC samples. A simple way to analyse expression in our cell lines would be to analyse mRNA or protein levels in both untreated and siRNA treated cells. This would allow for detection of any changes before and after treatment which may correlate with the effect on cell viability. Identification of siRNAs that affect OAC but not BO cell viability, or vice versa, has not been widely reported in oesophageal cell lines, although protein and mRNA analysis indicates variation in the levels of specific targets. For example, NET1 expression in oesophageal cell lines was shown to be significantly higher in OE33 OAC cells as compared to Het1A “normal” cells ($P=0.01$) (Lahiff et al., 2013). In breast cancer cell lines it was also shown that HMEC (“normal”) cell viability was greatly reduced (40%) after treatment with camptothecin as compared to MDA-MB-453 (5%) or MDA-MB-231 (15%) breast cancer cell lines (Abedin et al., 2007). A difference in cell proliferation was also noted between breast cancer cell lines themselves, for example treatment of T47D cells with camptothecin reduced cell viability by 30%, which may be due to the fact that the T47D cell line is oestrogen receptor positive, while both the MDA-MB-453 and MDA-MB-231 are oestrogen receptor negative. Gene expression within cell lines may also be dependent on specific signalling pathways, for example in gastric cancer (GC) it has been

demonstrated that, within a selection of six GC cell lines, COX-2 mRNA levels correlated with β -catenin expression highlighting a clear differential pattern (Nunez et al., 2011). These studies thus highlight the variation that can occur both between and within cell lines representative of varying malignancies. In relation to our findings, this verification process proved interesting, but we were more interested in the role of immune genes that decrease oesophageal cell growth significantly, rather than selective inhibition between cell lines spanning the carcinogenic sequence.

On review of the bioinformatics results and available literature, C1QA and LIF were chosen to carry forward for validation. TRPC7 is involved in calcium channel activity and was not chosen for further validation as it was not highlighted in DAVID or showed significance in Oncomine databases. While TRPC7 plays a major role in diabetes as a target for insulin secreting β -cell manipulation (Qian et al., 2002), it has no known link to cancer, although its role in B-lymphocytes has been identified. Although little is known about the role TRPC7 plays in cancer, calcium signalling has been implicated in several cancers, including lung and breast, as they can regulate cell proliferation, differentiation and migration (Choi et al., 2014, Wu et al., 2014b). In relation to oesophageal cancer, it has also been demonstrated that the use of mibefradil, a T-type calcium channel blocker, can reduce OSCC cell proliferation in the TE8 cell lines and also up-regulate p21 expression (Lu et al., 2008). One study has shown that TRPC7 plays a role in the formation of ion channels in B-lymphocytes, and appears to function as a phospholipase C-regulated, diacylglycerol-activated channel (Lievremont et al., 2005), thus it may be an interesting target for further investigation. Utilisation of Oncomine also illustrated that C1QA and LIF were upregulated in BO and OAC as compared to normal oesophageal tissue, whereas

TRPC7 remained unchanged in all oesophageal tissue types.. Further review of the bioinformatics results showed that LIF was identified in one of the IPA networks (Figure 4.9 & Figure 4.13). Leukaemia Inhibitory Factor (LIF) is a cytokine involved in the JAK/STAT pathway which is known to play a role in cancer cell progression, while complement component 1, subcomponent Q, A chain (C1QA), is a member of the first component of the serum complement system which plays a major role in innate immunity.

Innate immunity is the response of the host to defend itself against a pathogen which is present from birth before exposure to an antigen of pathogen, and it consists of cells and proteins that are always present and ready to mobilise at the site of infection or injury. The inflammatory microenvironment has recently been added as one of the hallmarks of cancer highlighting its important involvement in cancer onset. Mutations or genomic instability due to exposure to mutagens or cytokines can induce inflammation and the immune response. An example of the role of inflammation in cancer is the evidence that inflammation caused by ulcerative colitis (UC) increases risk of developing colorectal cancer (CRC). In 2001, the rate of progression to colitis-associated cancer from UC was identified as 3.7% (Eaden et al., 2001). In UC patients that do not respond to treatment, the loss of epithelial cells due to chronic inflammation, is compensated for by increased epithelial proliferation as a repair mechanism which may be uncontrolled and subsequently contribute to CRC. Mutations within p53 also play an important role in colitis-associated cancer (CAC) pathogenesis, with a deletion of p53 found in up to 85% of patients as reviewed by (Rogler, 2014). It was also demonstrated that a mutant form of p53 has gain-of-function activity which it exerts via augmentation and continued exposure of

epithelial cells to low amounts of the inflammatory cytokine TNF α . This resulted in continued NF κ B activation, in both pancreatic cancer and CRC cell lines (Cooks et al., 2013), concluding that p53 mutations may contribute to both tumour initiation and tumour progression. Another study illustrating the role of inflammatory cells in the release of tumour promoting cytokines was in a colitis-associated cancer (CAC) mouse model. This study indicated that inactivation of NF κ B in myeloid cells acted to reduce tumour growth and also inhibited the production of cytokines including IL-6 (Greten et al., 2004). A subsequent study demonstrated that the IL-6 cytokine mediates the effect of immune cells on CAC growth (Becker et al., 2004). Combined, these studies highlight the role of cytokines, released by immune cells, in the initiation of inflammatory related cancer. IL-6 has been identified as a key cytokine in inflammation and its role in the progression of several cancers has been demonstrated including head and neck, OAC, CAC and breast (Zhang et al., 2011, Yadav et al., 2011, Grivennikov et al., 2009, Berishaj et al., 2007).

From this evidence it has been suggested that IL-6 could be a target gene for cancer therapy and targeting of IL-6 is currently being tested in the treatment of both multiple myeloma and Castleman's disease. In multiple myeloma growth was inhibited using an anti-IL6 monoclonal antibody (mAb) ($p < 0.05$) which was also associated with inhibition of STAT3 (Fulciniti et al., 2009). In Castleman's disease, a lymphoproliferative disorder, a clinical study identified that 78% of patients reported a clinical benefit response following treatment with siltuximab, an anti-IL6 mAb (van Rhee et al., 2010). By inhibiting cytokines that mediate inflammation, this may block the activation of transcription factors such as NF κ B and STAT3.

Table 4.2: Twelve Immune Related Genes Used for Verification in Multiple Cell Lines

Gene Name	Role in Immune Response	Reference
CIQA	Component of the complement system which is activated during the immune response	(Fang et al., 2009)
TLR2	Activates intracellular signalling pathways resulting in NFκB activation and production of inflammatory and immune effectors	(Kim et al., 2013a)
LIF	A cytokine that activates STAT3 which in turn activates pro-oncogenic inflammatory pathways	(Fitzgerald et al., 2005)
SYMPK	Increased expression during tight junction formation; when dysfunctional, they are involved in activation of the immune response	(Chang et al., 2012)
TRPC7	Role in formation of ion channels in B-cells which are involved in the immune response	(Lievremont et al., 2005)
SON	Associated with MAPK pathway whose activation is involved in immune response	(Furukawa et al., 2012)
RUNX2	Activates PI3K/Akt pathway which plays a key role in innate immune response	(Tandon et al., 2014)
RAD51	Involved in DNA repair which can be inhibited by inflammatory cytokines	(Jaiswal et al., 2000)
KRT10	Involved in 2 inflammatory skin disorders; epidermolytic hyperkeratosis and ichthyosis with confetti	(Burger et al., 2012)
TRPV2	Specialized ion channel expressed in mammalian immune cells	(Santoni et al., 2013)
MNDA	Only found in nuclei of cells of the granulocyte-monocyte lineage (immune cells)	(Briggs et al., 1994)
MXD1	Part of the MAD/MAX/MYC family which are central regulators of inflammatory response	(Florea et al., 2013)

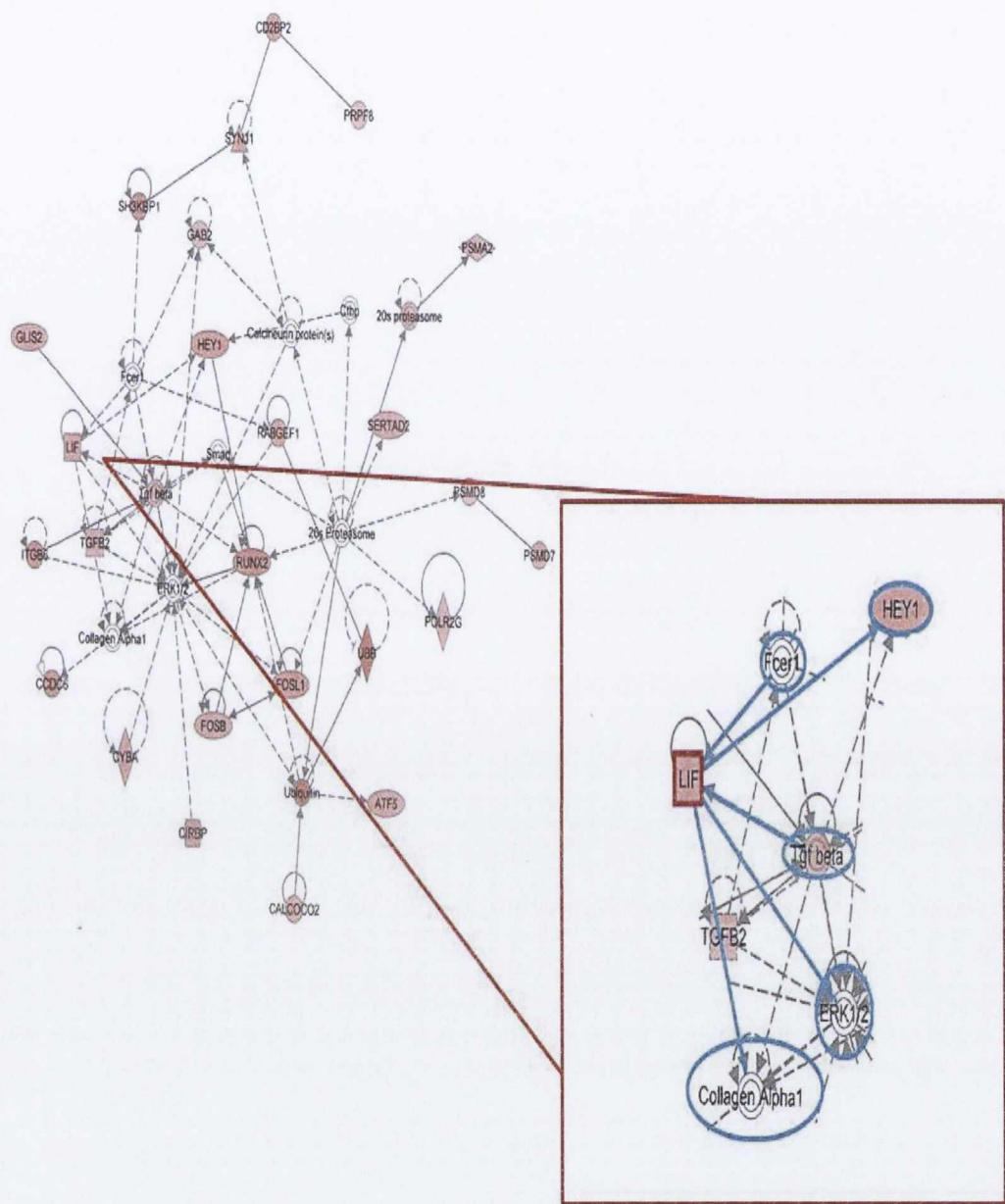


Figure 4.13: IPA Network Analysis Highlighting Gene Interactions with Leukaemia Inhibitory Factor (LIF). This is network 4 from figure 4.10, with the LIF connections highlighted. Data input for this analysis was cell viability results with Z-score < -1.6, SSMD < -5, P < 0.01 and CV < 40%. This pathway shows genes involved in cellular growth and viability, gene expression and cell-to-cell signalling and interaction.

The inflammatory microenvironment has recently been identified as a hallmark of cancer; therefore the identification of LIF as a regulator of OAC cell viability may be explained in these terms. The LIF receptor (LIFR) forms a heterodimer with gp130, to which LIF binds. The gp130 complex can also bind IL-6R resulting in tyrosine phosphorylation of JAK1 and JAK2. The SH2 domain of STAT3 can bind the phosphorylated tyrosine which in turn phosphorylates STAT3 at tyrosine residue 705 (Y705), resulting in its translocation to the nucleus (Wang et al., 2013b). It has been demonstrated that inflammatory cells in the tumour microenvironment, including macrophages, produce large amounts of IL-6 and other cytokines. It can also induce the release of C-reactive protein (CRP), thus can act as an autocrine growth modulator (Grobewska et al., 2012). For these reasons, LIF is an interesting candidate for further functional validation within the STAT3 pathway.

C1QA is a member of the serum complement system which plays a key role in innate immunity. Complement proteins within the plasma are usually inactive, but under certain conditions they can be activated. C1q acts to recognise immune signals which results in proteolytic activation of C1r and C1s, which can trigger the activation of the classical complement pathway. C1QA is one component of C1q, along with C1QB and C1QC, and we have shown that this one small component of C1q and the complement system can modulate OAC cell viability. A recent study has shown that C1q induced a significant increase in cytosolic β -catenin and can activate the canonical Wnt signalling pathway to promote cellular processes associated with mammalian ageing (Naito et al., 2012). Aberrant canonical Wnt signalling can also contribute to carcinogenesis, and has been implicated in the pathogenesis of BO

(Clément et al., 2007, Moyes et al., 2012, Bian et al., 2000). Given this newly identified possible association between Wnt signalling and C1q, functional validation of C1QA will be undertaken to see the effect of C1QA knockdown on β -catenin localisation and the Wnt signalling pathway within the oesophageal cancer setting.

This is the first time a siRNA library screen has been carried out in OAC cells; therefore it was hypothesised that novel results would be generated. An effective and reliable screening method was developed and utilised to analyse the effect of the 6,022 siRNAs contained in the druggable genome library on OAC cell viability. The impact of these results raises many questions about the relationship between inflammation, innate immunity and oesophageal cancer. This screen has also highlighted numerous possible avenues of investigation, which goes far beyond what is described within this body of work. Currently work is being undertaken by other members of the team to validate different hits identified by the screen highlighting the scope of what has been discovered.

Chapter 5

Results

*LIF and C1QA Mediate Oesophageal
Cancer Cell Survival Through STAT3
and β -catenin Signalling Pathways*

5 LIF and C1QA Can Mediate Cancer Cell Survival via STAT3 and β -catenin Signalling

5.1 Introduction

The availability of pooled siRNA libraries has enabled the implementation of many genome wide screens in mammalian cells including breast and ovarian cancers (Hu et al., 2012, Sethi et al., 2012). Pooling siRNAs allows for high specificity, by several different sequences to target the specific gene of interest, but at a low concentration so as not to induce a cytotoxic effect (ThermoScientific, 2013, Caffrey et al., 2011). Functional validation of hits generated by siRNA screens is essential to confirm specificity of the siRNA to the gene of interest rather than skewing data with sequence-independent or sequence-specific off-target effects. This can be performed using a rescue experiment which is the incorporation of the recombinant protein to rescue the phenotype being analysed, whether it be a signalling event or as in this case, an effect on cell proliferation. This is performed by reintroducing a version of the target gene which cannot be silenced by the presence of the siRNA (Jackson and Linsley, 2010, Kittler et al., 2007, Echeverri et al., 2006)

Other than rescue experiments, which are considered the gold standard of RNAi validation, a number of other quantitative controls should be used. There are many different levels at which validation of knockdown should be confirmed; firstly, gene knockdown should also be confirmed at mRNA level to verify siRNA mediated knockdown. Another simple method to validate knockdown of a specific gene is to measure the protein expression preferably with Western blot. If protein expression is shown to correlate with the phenotype, then it can be deemed that it is gene-specific.

If the hits identified are transcription factors, another method of validation is to analyse their effect on transcription of relevant genes within the biological pathway. For example, if IL-6 is identified as a hit it should be validated via its role in STAT3 activation (Wang et al., 2013b).

Leukaemia Inhibitory Factor (LIF) is a secreted protein that is part of the IL-6 family of cytokines. When it binds to its receptor, LIFR, it induces tyrosine phosphorylation and forms a heterodimer with the common transmembrane receptor subunit, gp130 (McColm et al., 2006), upon which it subsequently activates the JAK/STAT pathway. The gp130 receptor is a ubiquitously expressed signal-transducer receptor which forms a receptor complex for several IL-6 family cytokines; IL-6, LIF, OSM, CNTF and CT-1 (Figure 5.1), and initiation of gp130 signalling is mediated by the binding of these cytokines. Activation of the gp130 complexes triggers activation of the JAK/STAT pathway, by phosphorylation of Jak1 and Jak2 and subsequently STAT, and MAPK pathway, by SHP2 recruitment (Stahl et al., 1994, Ogata et al., 1997).

LIF is produced by epithelial cells during inflammation and infection (Knight, 2001), and is most widely studied in relation to development and embryonic stem (ES) cells. In reproductive biology, LIF plays a key role in trophoblast cell adhesion, proliferation and invasion via the STAT3 pathway (Suman et al., 2013b). In human myoblasts, siRNA mediated knockdown of LIF receptor (LIFR) decreased mRNA levels by 50% ($p < 0.05$), but also significantly decreased proliferation ($p < 0.05$) which suggests that LIF may regulate myoblast proliferation in an autocrine manner, while addition of recombinant LIF subsequently significantly increased cell proliferation ($p < 0.05$) (Broholm et al., 2011). In relation to carcinogenesis, it has been reported that

LIF can promote cell proliferation in human cancer cells, including breast, stomach, liver, colon, lung and pancreas, via expression of c-fos, jun-B and cyclin-E (Kamohara et al., 1994). Within the inflammation setting high LIF levels are present in ulcerative colitis patients (Guimbaud et al., 1998), while it has also been shown that LIF can either be induced by or can itself induce, cytokines which promote pancreatic tumour growth, as indicated by analysis of inflammatory cytokines including TNF α , IL-1 β , IL-6, IL-8, and LIF, resulting in induction of endogenous LIF mRNA (Kamohara et al., 2007).

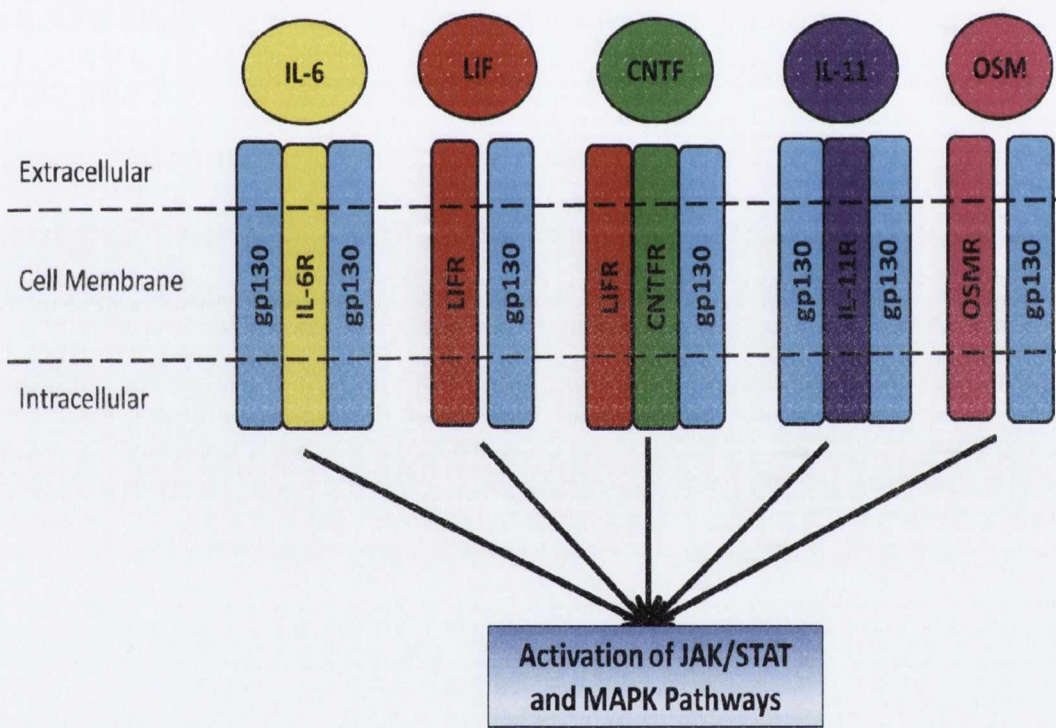


Figure 5.1: Cytokine Receptor Complexes Utilising gp130. Various combinations of receptors are used for pathway activation. The gp130 homodimers associate with IL-6R. LIF binds to the heterodimer of LIFR and gp130. This heterodimer can also associate with CNTF and CT-1. OSMR forms a heterodimer with gp130. The signal-transducing subunit gp130 is found within all complexes. It is responsible for the intracellular activation of the Janus-activated kinase–signal transducer and activator of transcription (JAK–STAT) and the mitogen-activated protein kinase (MAPK) pathways (Modified from (Bauer et al., 2007)).

As previously outlined, LIF signals through the JAK/STAT pathway to exert its biological effects, and STAT3 is thus phosphorylated by JAK resulting in its activation, initiating both cytoplasmic accumulation and its subsequent translocation to the nucleus. It is generally expressed at a basal level in cells, but upon activation by cytokines, levels increase significantly, which has been demonstrated in OSCC tissues resulting in high levels of Oct-1, a ubiquitous transcription factor which can either activate or repress the transcription of several other genes (Wang et al., 2013c). Constitutive activation of STAT3 can also impact on angiogenesis, as it can result in activation of the VEGF promoter indicating that STAT3 directly regulates VEGF and thus growth, angiogenesis and metastasis in pancreatic cells (Wei et al., 2003). Within the inflammatory response, STAT3 can be triggered by cytokines including IL-6 (Picardo et al., 2012, Korkaya et al., 2012), which is a pro-inflammatory cytokine contributing to malignant transformation through activation of the NF κ B pathway. In turn, this initiates constitutive production of both IL-6 and IL-11, thus generating a positive feedback loop (Iliopoulos et al., 2011, Schafer and Brugge, 2007). Although, IL-8 has been implicated in this positive feedback loops by activating NF κ B, studies have thus far shown that it is only maintained by IL-6 and IL-11.

Constitutive activation of STAT3 at tyrosine phosphorylation at residue 705 has been observed during oesophageal adeno-carcinogenesis, for example phosphorylated STAT3 (pSTAT3) staining levels are increased within the nuclei of dysplastic BO and OAC samples, with increasing median IHC staining scores from squamous epithelium through the oesophageal sequence to OAC (Dvorak et al., 2007). In this study IL-6 mRNA was increased significantly following exposure to low pH and/or bile acids, with the combination of both showing the greatest increase and correlating with

increased immunofluorescent nuclear staining of pSTAT3. A more recent study also demonstrated IL-6 dependant pSTAT3 activation in transformed BAR-T cells, with inhibited p53 and expressed H-RAS^{G12V}, that was not observed in non-transformed BAR-T cells (Zhang et al., 2011). Inhibition of STAT3 using known STAT3 inhibitors has already shown success in tumour size reduction in several cancers including colitis-associated cancer and skin cancer (Bollrath et al., 2009, Chan et al., 2004). Due to complications using STAT3 inhibitors, including development of Th1-associated autoimmunity in mice (Kortylewski et al., 2005), targeting the IL-6 family of cytokines may not result in these undesired affects. Therefore, therapeutic targeting of the STAT3 pathway via members of the IL-6 family of cytokines including LIF may be an interesting prospect.

The main biological function of the complement system is the recognition and destruction of invading micro-organisms, resulting in activation of the innate immune response. Due to its ability to recognise non-self elements, it is postulated that it should also recognise tumour cells, but it has been found that tumour cells can over-express complement regulatory proteins, including CD59, CD46 and CD55, which may help to avoid complement mediated cell lysis (Gancz and Fishelson, 2009, Fishelson et al., 2003). Complement component 1, subcomponent Q, chain A (C1QA) is a constituent of C1q subcomponent, which is itself a subcomponent of C1 within the complement system. C1q acts as a recognition signal for immune complexes which initiates the classical complement pathway and binds to C1r and C1s to form C1, which is the first component of the classical pathway of the complement system. Other effects of complement activation include; degranulation of basophils, neutrophils and mast cells, the release of neutrophil products such as elastase and

oxygen radicals, and extracorporeal blood circulation (Parry et al., 2000, Golden et al., 1987, Andoh et al., 2001, Wanscher et al., 1989, Videm et al., 1988).

Innate immune factors, including cytokines, have been increasingly observed to contribute to cancer development in multiple studies as previously outlined. For example, *H. pylori* infection is associated with gastric cancer (Cavaleiro-Pinto et al., 2011, Damania et al., 2014), and a significant increase ($p < 0.05$) in plasma TNF and IL8 levels were demonstrated in MALT lymphoma patients as compared to controls (Konturek et al., 2000). However, the contribution of complement components as a characteristic of innate immunity has been less well detailed in relation to cancer as it is generally known for its role in facilitating cancer cells to avoid apoptosis. However, C1q has been demonstrated to initiate sustained activation of WOX1, a tumour suppressor gene, resulting in cell apoptosis in prostate cancer development (Hong et al., 2009) and this study also showed that downregulation of C1q increased prostate hyperplasia and tumour formation due to reduced WOX1 activation. Another more recent study has also identified that during wound healing, C1q is deposited in endothelial cells highlighting its role as a possible angiogenic factor (Bossi et al., 2014). Specific C1QA polymorphisms such as C1QA₂₇₆ have also been associated with lymphoma and breast cancer development. Furthermore, this polymorphism may be used as a potential marker for response to R-CHOP chemotherapy treatment in lymphoma (Jin et al., 2012), which is indicative of reduced haematogenous spread of breast cancer (Racila et al., 2006).

In BO and OAC, translocation of β -catenin to the nucleus has been observed in the carcinogenic sequence; furthermore Wnt activation induces oesophageal hyperplasia

in murine models (Moyes et al., 2012, Clement et al., 2006a). Targeting the Wnt pathway has already shown positive results in NSCLC and colon cancer, by siRNA silencing of Dishevelled (Dvl) and β -catenin (Verma et al., 2003, Uematsu et al., 2003), while radiofrequency ablation of BO cells within the oesophagus has also resulted in increased levels of phosphorylated β -catenin inducing a repair response (Krishnan et al., 2012). A recent study suggested that C1q may also activate Wnt signalling by binding to Frizzled (Fz) receptors which in turn induces cleavage of LRP6, a protein known to be involved in Wnt activation as indicated by increased cytosolic β -catenin with C1q treatment (Naito et al., 2012). Given the important role the Wnt/ β -catenin pathway plays in BO and OAC, analysis of C1QA within this setting may thus be of particular interest.

5.2 Hypothesis and Objectives

The question is thus posed; what are the mechanisms through which LIF and C1QA exert their cellular effects on oesophageal cell viability, and how do they thus contribute to oesophageal carcinogenesis? These aims were achieved through:

- Confirmation of LIF and C1QA silencing at the mRNA and protein levels by RT-PCR and Western blotting and confocal
- Reintroduction experiments involving use of both recombinant and native proteins to demonstrate specificity of the siRNAs and to demonstrate signalling pathways responsible for the observed effects
- Examination of STAT3 activation status following LIF inhibition/re-introduction/treatment by Western blot and by immunofluorescent localisation of pSTAT3
- Analysis of STAT3 target gene (IL6) expression induced by LIF
- Analysis of β -catenin localisation and stabilisation in cytoplasm and nucleus following C1QA silencing
- Utilisation of TCF/LEF luciferase reporter assays to analyse the effect of C1QA inhibition on Wnt signalling pathway
- Implementation of a small siRNA screen to determine if synergistic or amplified responses to combined inhibition of a pre-defined '*immune panel*' would occur when co-silenced with either LIF or C1QA

5.3 Results

5.3.1 Leukaemia Inhibitory Factor (LIF)

5.3.1.1 Confirmation of LIF Knockdown at mRNA and Protein Levels

In order to confirm specific LIF silencing, following transfection with siRNA targeting LIF expression, GOhTRT high grade dysplastic cells and SK-GT-4 oesophageal adenocarcinoma cells, were seeded at 5×10^3 and 7.5×10^3 cells/well respectively, in a 12-well format, and reverse transfected as per sections 2.3.

Figure 5.2 indicates LIF mRNA silencing was highly significant compared to non-targeting siRNA treated cells, in the GOhTRT cells line with a reduction of $77\% \pm 2$, and SK-GT-4 cell line with a reduction of $55\% \pm 6$ ($p < 0.0001$). This supports the finding that a reduction in cell viability in the GOhTRT cells lines, seen during the screen and verification steps, was due to siRNA mediated LIF silencing. Furthermore, the RT-PCR results shows that LIF is also present in the OAC cell line, SK-GT-4, and that LIF siRNA can reduce this level significantly.

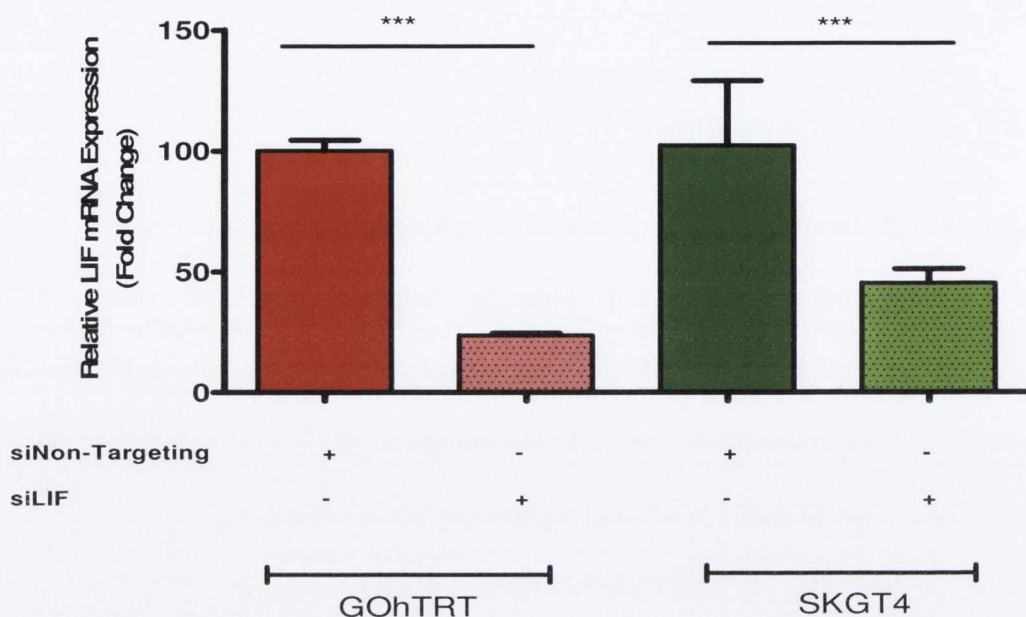


Figure 5.2: LIF Knockdown at mRNA Level. LIF mRNA expression decreased by $77\% \pm 2$ in the high grade dysplastic GOhTRT cell line, while in the OAC cell line SKGT4, LIF mRNA expression decreased by $55\% \pm 6$. $N=3$, and represent mean $\pm$ SEM (t-test; *** $p<0.0001$).

Confirmation of LIF knockdown was also necessary at a protein level, utilising both Western blot and immunofluorescence staining, using a LIF polyclonal antibody (Sigma Aldrich). For Western blot, GOhTRT cells were seeded at 35×10^3 cells per well in a 6-well plate as per section 2.11, and for immunofluorescent staining, cells were seeded at 5×10^3 , in a 96-well plate as per section 2.13. Each condition designed in pooled triplicate wells. Conditions were; untreated, non-targeting siRNA treated, LIF siRNA treated, or LIF siRNA treated followed by treatment with exogenous recombinant LIF protein. Figure 5.3A shows confirmation of siRNA mediated LIF knockdown by Western blotting, indicating a 50.5% knockdown compared to non-targeting siRNA treated cells after normalisation to the loading control (β -actin) per sample. This was further confirmed by immunofluorescent staining of the GOhTRT

cells, with a significant decrease in LIF staining noted in siLIF transfected cells ($p < 0.05$) as compared to the non-targeting negative control (Figure 5.3B). Western blotting and immunofluorescent staining experiments performed as above demonstrated specific silencing of LIF expression (Figure 5.3), occurring during the observed loss of cell viability (Figure 4.14).

5.3.1.2 Exogenous Recombinant LIF Treatment Reverses Loss of Cell Viability

Following siRNA Mediated LIF Silencing and Increases Intracellular LIF mRNA and Protein Levels.

The aim of this section was to assess the effect of exogenous recombinant LIF (R&D Systems), when added to cells which had undergone LIF inhibition by siRNA. Restoration of cell viability in this context would indicate that these observations were mediated by LIF induced signalling events. Therefore, reverse transfection in the GOhTRT cells, of the non-targeting or LIF siRNAs was facilitated by DharmaFECT4 transfection reagent at; 0.2 μ L for MTT assay, 2 μ L for RT-PCR and 5 μ L for Western blot, per well. At 48 hours post transfection, 30 ng/mL exogenous recombinant LIF (R&D Systems) was added to corresponding wells. At 96 hours post-transfection (final time point/end of experiment), one plate was analysed for effect on protein levels via Western blot (Figure 5.3A), the second plate was to detect effect on cell proliferation via MTT assay (Figure 5.4A), while the final plate was used to detect mRNA expression via RT-PCR (Figure 5.4B). All experiments were performed in triplicate wells for triplicate biological replicates.

As previously outlined, transfection of the GOhTRT cells with siLIF can result in effective silencing of LIF protein (Figure 5.3A). However, treatment of siLIF transfected cells with recombinant LIF resulted in restoration of intracellular levels of

LIF protein to that of the non-targeting siRNA treated cells. This indicates that exogenous LIF signalling may result in increased cytoplasmic LIF production. Immunofluorescent staining (Figure 5.3B), confirmed that treatment with exogenous recombinant LIF significantly reversed the observed reduction in LIF intracellular staining as compared to the non-targeting negative control treated cells ($p < 0.05$). MTT assay results (Figure 5.4A) indicated that siRNA mediated knockdown of LIF resulted in a significant decrease in cell viability of $38\% \pm 2.1$ vs non-targeting ($p < 0.05$), which was reversed by treatment with exogenous recombinant LIF protein ($91\% \pm 2.2$ vs non-targeting). Additionally, Figure 5.4B shows a reduction ($79.5\% \pm 7.8$ vs non-targeting) in mRNA levels following treatment with siLIF, which was again reversed following treatment with exogenous recombinant LIF, restoring levels back to that observed in resting cells.

In all, we can conclude that siRNA mediated LIF knockdown is specific and furthermore, that exposure to exogenous recombinant LIF can increase the intracellular levels of LIF in an autocrine manner. This has previously been demonstrated in Hek293 cells by their uptake of exogenous human PDCD5 protein which was added to the growth medium of the cells (Wang et al., 2006), suggesting that PDCD5 is avidly taken up by Hek293 cells via clathrin-independent endocytosis. In conclusion, results indicate that LIF signalling may form an autocrine signalling loop in oesophageal cells.

A



B

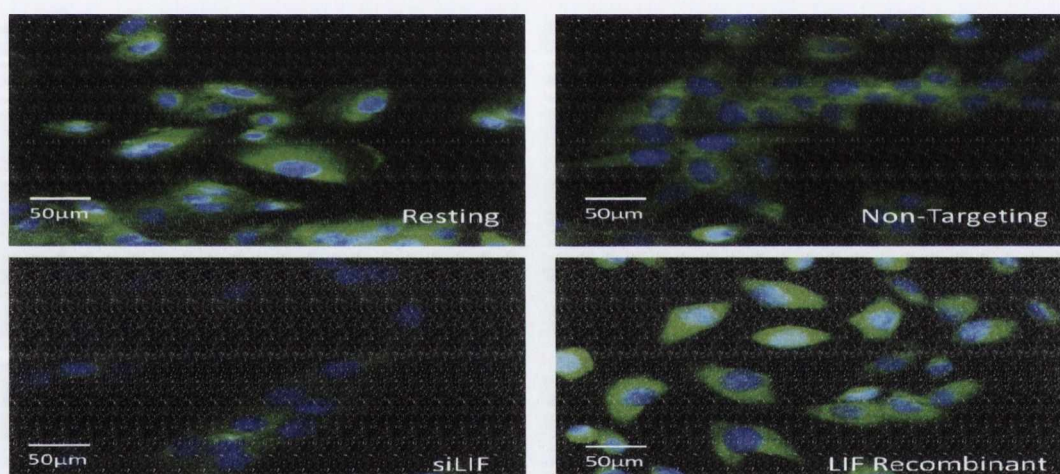


Figure 5.3: Confirmation of LIF Silencing at a Protein Level Which is Reversed upon Treatment with Recombinant LIF. siRNA mediated LIF silencing has been confirmed by Western blot and immunofluorescent staining, which could be reversed by treatment with recombinant LIF. Untreated GOhTRT cells functioned as a positive control for the anti-LIF polyclonal antibody as basal levels for the cell line had already been confirmed. A) Western blot analysis using the LIF antibody indicating successful LIF silencing and reversal when treated with LIF siRNA and recombinant LIF in which β -actin served as a loading control, B) The inhibition and reversal observed in the Western blot was also confirmed by immunofluorescent staining. Images were taken at 10x magnification. N=3. Western blot densitometry analysed using t-test (** $p < 0.01$, *** $p < 0.0001$)

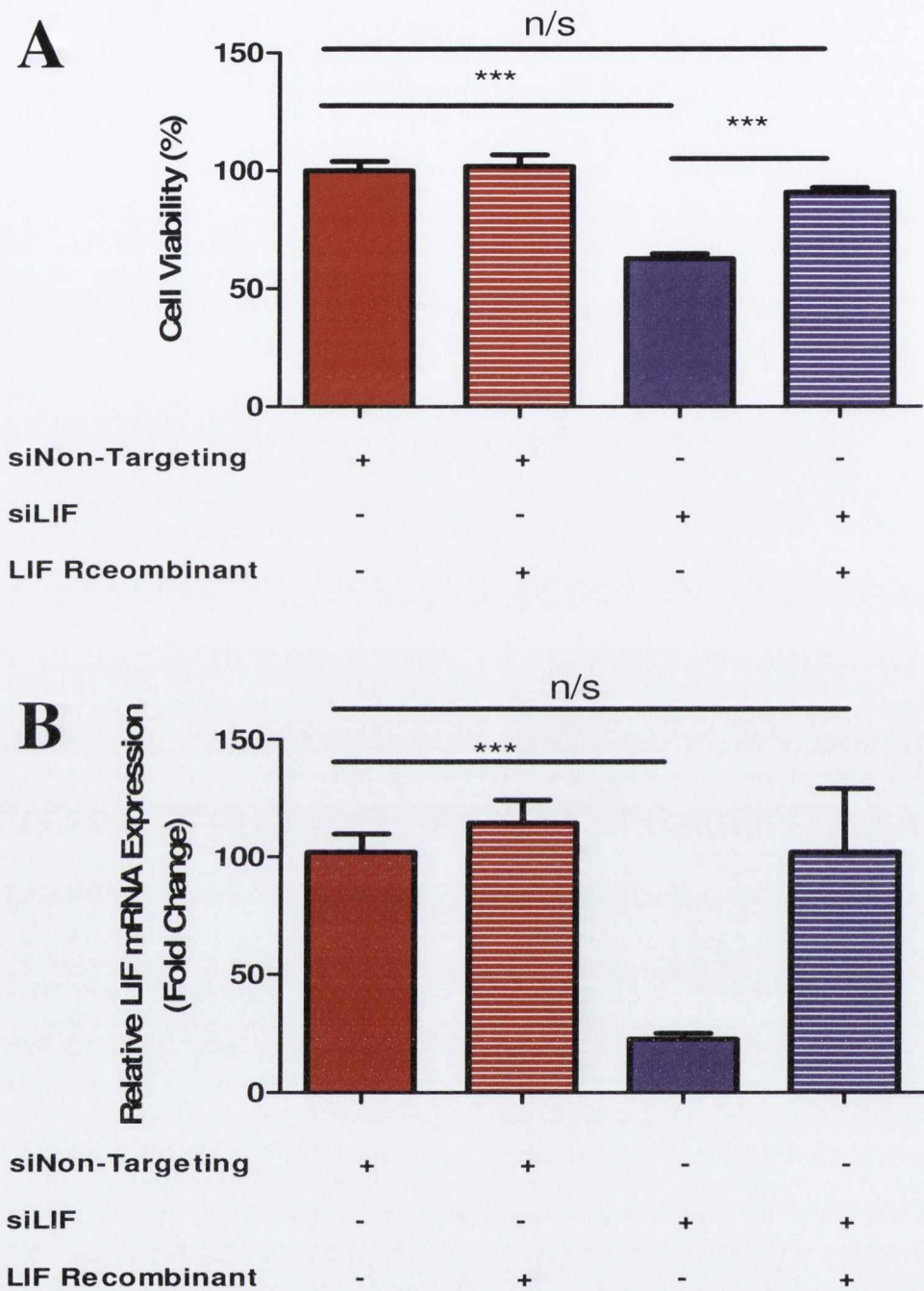


Figure 5.4: Reintroduction of Recombinant LIF Reverses Observed Decrease in Cell Viability and LIF mRNA Level. A) MTT assay indicates a reduction in cell viability after treatment with LIF siRNA which was reversed when combined with exogenous recombinant LIF protein, B) RT-PCR indicating reduction of LIF mRNA using LIF siRNA, and recovery of the LIF mRNA after treatment with exogenous LIF protein. N=3, represented by mean $\pm$ SEM. (n/s: not significant, t-test; *** $p < 0.0001$)

5.3.1.3 Detection of Secreted LIF Levels in Cell Culture Supernatant

An ELISA immunoassay to determine the levels of secreted LIF in four cell lines 48hrs after seeding was implemented. Each cell line was seeded in a 24-well plates at 2.5×10^3 cells per well as per section 2.15, in duplicate biological replicates, with duplicate ELISA wells for each sample. The HET1A cells represent normal oesophageal cells, GOhTRT represent HGD, SK-GT-4 represent OAC and HepG2 cells are used as a positive control, as they are known to constitutively secrete LIF (Mezzasoma et al., 1993). The experiment was repeated in duplicate for each cell line, with each experiment analysed in duplicate again in the ELISA. Figure 5.5 shows the varying levels of secreted LIF within each cell line. As can be seen, there is a steady increase in secreted LIF from normal oesophageal (154pg/mL) to HGD (219pg/mL) to OAC (300pg/mL), with the increase between the HET1A and SK-GT-4 showing statistical significance ($p=0.044$). The positive control HepG2 indicates a level of 241pg/mL, which is lower than that of the OAC cell line used. Ultimately, this data indicates that OAC cells secrete large amounts of LIF, and more than HET-1A cells which represent normal squamous tissues. The limitation associated with this finding is that the results were not normalised to the cell number, which may contribute to the differing levels of secreted LIF. The cell numbers were calculated to allow for sustained cell growth, but a cell count was not preformed following incubation before the supernatant was removed. Although the cells were incubated for the same period of time, the rate of cell growth may differ significantly, thus impacting on the level of LIF protein secreted from the cells. For example, the recommended sub-cultivation ratio advised for the HET1A cell line is 1:3 to 1:4, while for the GOhTRT cell line the ratio is 1:3 to 1:6 indicating that the GOhTRT cell line may proliferate faster than the HET1A cells (ATCC, 2015). Further investigation of this finding, at other selected

time-points or using clinical samples, combined with normalisation to the cell number, may prove of interest.

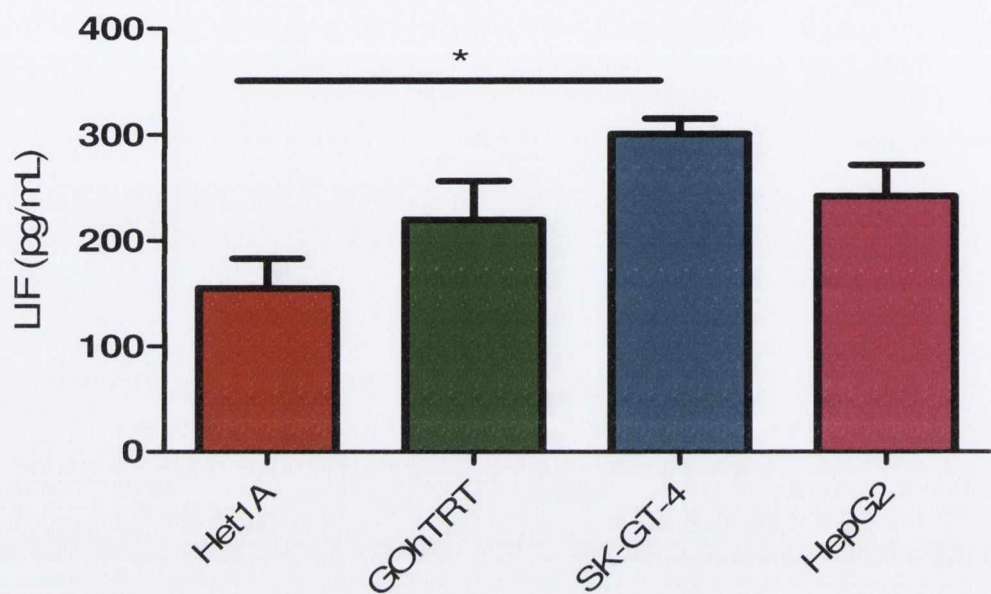


Figure 5.5: ELISA Immunoassay for Detection of Secreted LIF in Four Cell Lines. The ELISA to detect human secreted LIF was setup in 4 cells lines to analyse the varying level of secretion across the oesophageal carcinogenic sequence from normal oesophageal (HET1A), to HGD (GOhTRT), to OAC (SK-GT-4). Results showed a significant increase in secreted LIF between HET1A and SK-GT-4 cells ($P=0.044$). The level of secreted LIF was also shown to be higher in the SK-GT-4 cell line compared to the HepG2 cells which were used as a positive control. $N=3$, and again analysed in duplicate ELISA wells. Results represent mean $\pm$ SEM and analysed using the t-test ($*p<0.05$).

The above experiments (Figure 5.3&Figure 5.4) demonstrated that exogenous LIF may induce the expression of its own transcript and that it may be secreted by OAC cells. To determine whether secreted levels of LIF are also reduced following siRNA mediated inhibition of LIF, cells were seeded at 2.5×10^3 cells per well in a 24-well plate and reverse transfection was setup as per section 2.3. Briefly, non-targeting or LIF siRNA was transfected into the GOhTRT cells using 1.4 μ L DharmaFECT 4 per

well, giving a final siRNA concentration of 25nM and incubated for 96 hrs. The ELISA was then performed as per section 2.15, in duplicate biological replicates, with duplicate ELISA wells for each sample. To control for any non-specific binding due to the presence of 5% FBS in the cell culture media, media without cells was also incubated and analysed via ELISA, with results indicating levels less than 5pg/mL. The cell supernatant was removed at 96-hrs post-transfection and centrifuged to pellet any particulates. Results confirmed that siRNA mediated LIF knockdown reduces secreted human LIF significantly (1.347pg/mL), as compared to untreated cells (1826pg/mL) ($p<0.001$) and non-targeting siRNA treated cells (1685pg/mL) ($p<0.01$).

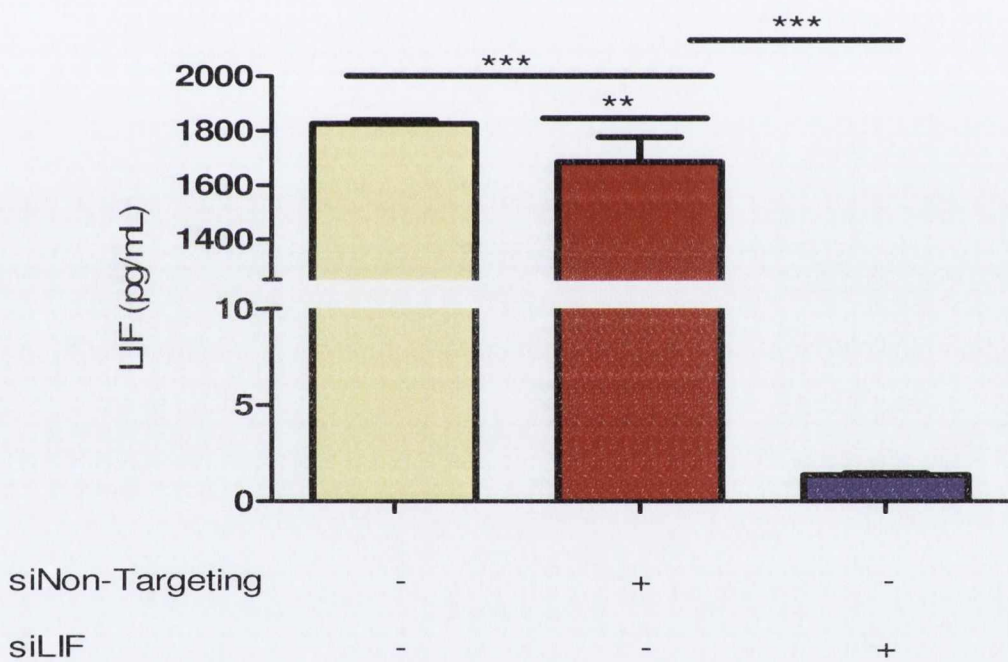


Figure 5.6: ELISA Immunoassay Confirms siRNA Mediated Knockdown of LIF Also Inhibits LIF Secretion. A human ELISA kit was used to analyse effect of siRNA mediated LIF silencing on secreted LIF. Cells were treated with the corresponding siRNA and media containing 5% FBS was also analysed to control for any non-specific binding due to the presence of FBS. This indicated <5pg/mL. N=3 and analysed in duplicate ELISA wells. Results represent mean $\pm$ SEM (t-test; ** $p<0.01$, *** $p<0.001$).

5.3.1.4 STAT3 Activation is Reduced Following LIF silencing and recovered by Recombinant LIF

Binding of Il-6 family members to their corresponding receptors is known to result in STAT3 activation, therefore we hypothesised that LIF would also signal through this pathway. Furthermore, it was postulated that inhibition of LIF may also prevent STAT3 activation. Therefore, STAT3 activation status was examined following siRNA mediated gene silencing of LIF in the GOhTRT cells by Western blot and immunofluorescence as previously described. Briefly, cells were seeded at 35×10^3 per well in a 6-well plate and reverse siRNA transfection was facilitated by addition of 5 μ L DharmaFECT 4 transfection reagent per well. At 72hrs post-transfection, exogenous recombinant LIF protein (30ng/mL) was added to the corresponding wells and protein was then extracted at 96hrs post-transfection. Utilisation of β -actin served as a protein loading control. Immunofluorescent imaging was carried out as previously described. Briefly, for the GOhTRT cells, 5×10^3 cells per well were seeded in a 96-wells plate. Cells were reverse transfected using 0.2 μ L DharmaFECT4 to facilitate siRNA transfection. At 72hrs post-transfection, corresponding cells were treated with 30ng/mL and at 96hrs post-transfection cells were fixed with 8% PFA. Cells were stained with pSTAT3 monoclonal antibody and Hoechst 33342 was used for nuclear staining. Images were taken using the GE Healthcare InCell analyser at 20x magnification.

Figure 5.7A shows a significant decrease in pSTAT3 of 60% following treatment with LIF siRNA ($p < 0.05$), which was reversed upon treatment with exogenous recombinant LIF protein. Variations in loading controls were corrected for using densitometry. Figure 5.7B confirms both inhibition of STAT3 activation following

LIF siRNA treatment, and the subsequent activation following treatment with exogenous recombinant LIF. Thus, Western blotting and immunofluorescent experiments demonstrate that siRNA mediated LIF silencing results in reduced the levels of pSTAT3 (Y705) within the GOhTRT cells. Additionally, treatment of LIF silenced cells with recombinant LIF protein resulted in reactivation of pSTAT3 further supporting the hypothesis that STAT3 phosphorylation at residue 705 is LIF dependant and that constitutive LIF production may be STAT3 mediated postulating a dysregulated feedback loop in OAC cells.

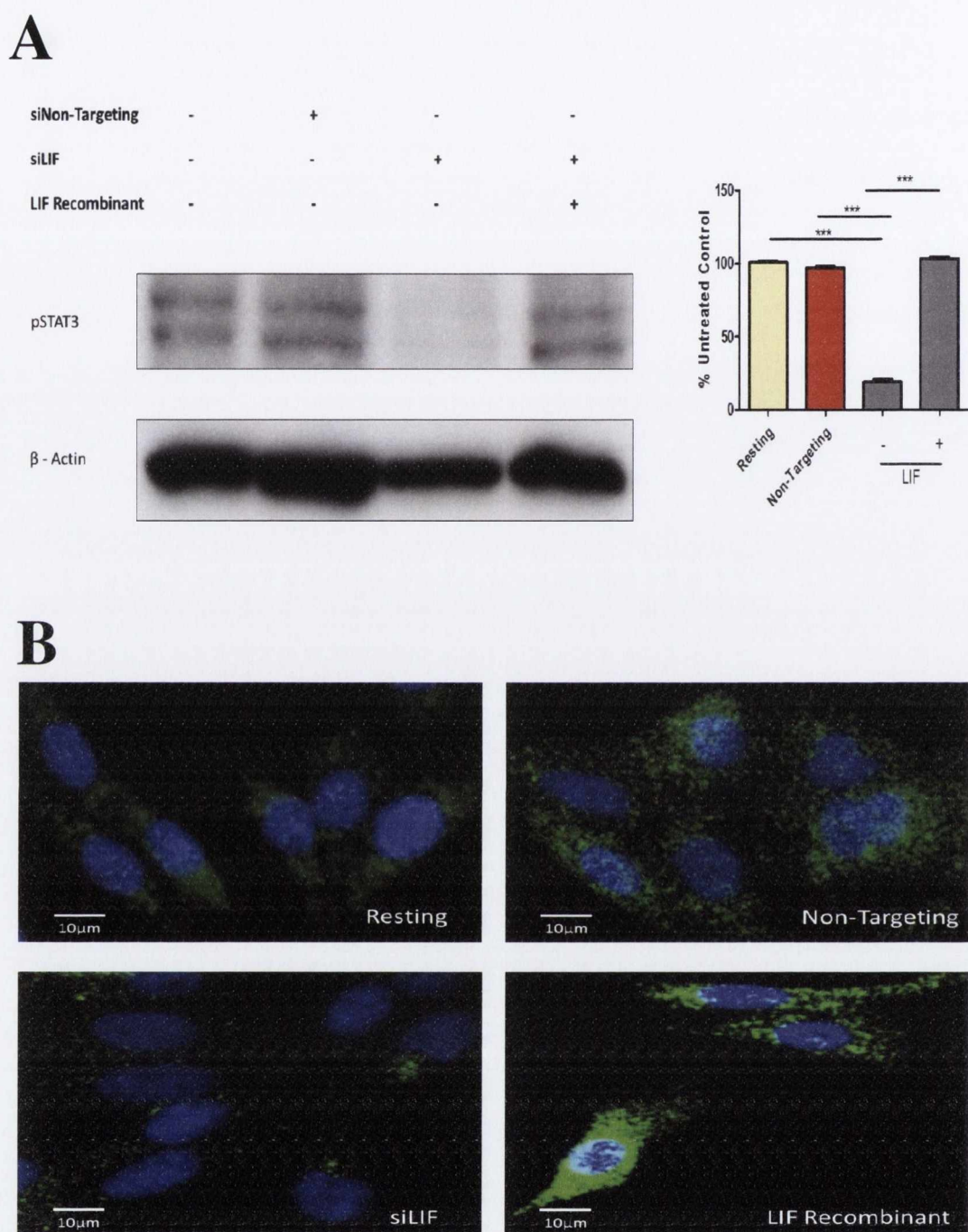


Figure 5.7: STAT3 Phosphorylation is Inhibited Upon siRNA Mediated LIF Knockdown. A) Western blot analysis shows a statistically significant decrease in pSTAT3 following treatment with LIF siRNA. B) Immunofluorescent imaging confirms a decrease in pSTAT3 within LIF siRNA treated cells. Images were analysed at 20x magnification. N=3. Western blot densitometry was analysed using the t-test (** $p < 0.0001$).

5.3.1.5 LIF Knockdown Reduces IL-6 mRNA Levels which are recovered Using Exogenous Recombinant LIF Protein

To further elucidate the mechanism through which LIF silencing affects STAT3 activation, RT-PCR was utilised to detect IL-6 mRNA levels (a STAT3 target gene) following various treatments, for evidence of redundancy between both LIF and IL-6. Redundancy has previously been highlighted between other members of the IL-6 family in particular between LIF and OSM (Gearing et al., 1992), in which OSM inhibition did not affect LIF levels, but three publications have suggested that redundancy may not exist between LIF and IL-6 (Zhu et al., 2001, Sugiura et al., 2000, Tofaris et al., 2002), indicating that one may regulate the other. Thus, to further investigate this in the oesophageal setting, GOhTRT cells were seeded at 2.5×10^3 cells per well in a 24-well plate, followed by transfection with LIF siRNA which was reverse transfected into the cells with 1.4 μ L DharmaFECT4 transfection reagent. At 48hrs post-transfection, corresponding wells were either treated with 30ng/mL exogenous recombinant LIF (R&D Systems) or 50ng/mL exogenous recombinant IL-6 (Peprotech). GAPDH was used as an endogenous control and results were normalised to non-targeting siRNA treated cells.

Figure 5.8 shows that IL-6 mRNA levels are reduced by $80\% \pm 0.66$, upon inhibition of LIF, while treatment with both exogenous recombinant IL-6 and LIF significantly blocked this reduction ($p < 0.0001$). BLAST analysis of the LIF siRNA sequences did not show any sequence similarities between LIF and IL-6. This suggests that LIF may regulate IL-6 expression through STAT3 activation in oesophageal cells, indicating that redundancy may not occur between these two IL-6 family members.

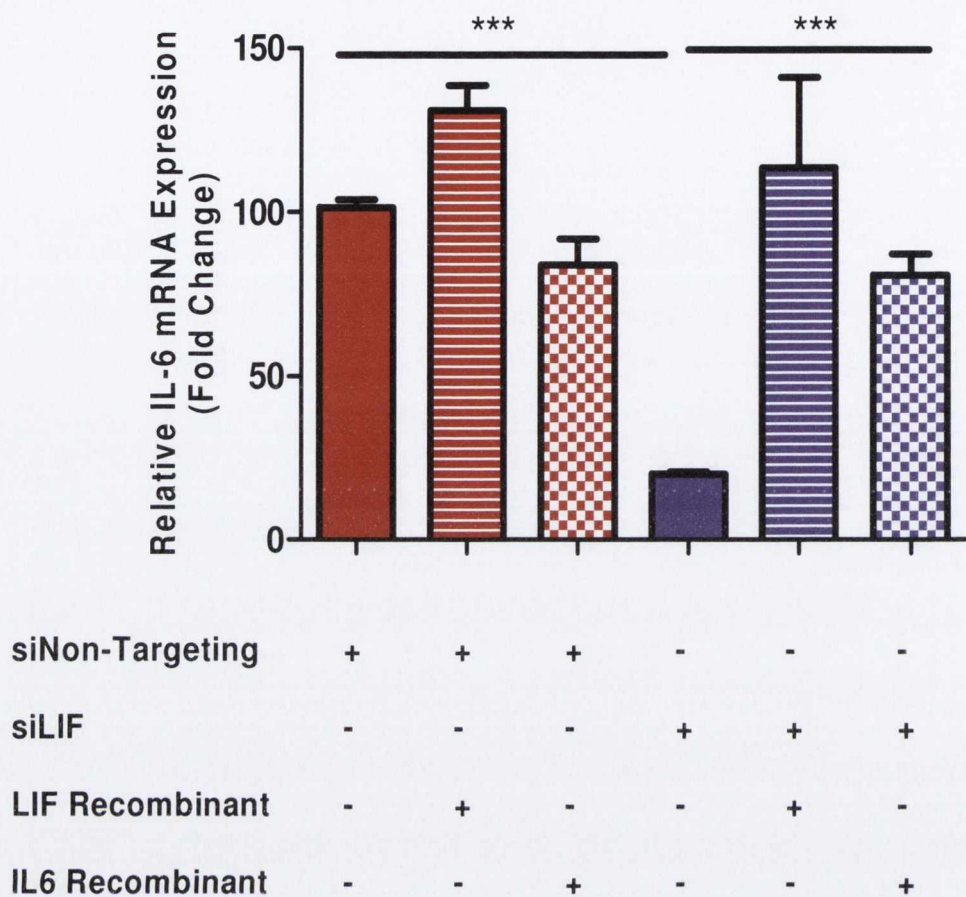


Figure 5.8: Treatment with LIF siRNA Reduces IL-6 Levels Significantly. To study effect of LIF knockdown on IL-6 levels, gene expression was analysed using RT-PCR. Results show a reduction in IL-6 expression ($80\% \pm 0.66$) when LIF is silenced via siRNA. When cells were treated with IL-6 or LIF exogenous recombinant proteins, IL-6 expression was recovered by 60% and 93% respectively. N= 3 and results represent mean $\pm$ SEM with significance detected using the t-test (** $p < 0.0001$).

5.3.2 Complement Component 1, Q Subcomponent, A Chain (C1QA)

5.3.2.1 Confirmation of C1QA Knockdown at mRNA and Protein Levels

As with LIF, siRNA mediated silencing of C1QA must be verified at both mRNA and protein levels, by RT-PCR, and Western blot and immunofluorescence respectively. Real time PCR was used to analyse mRNA knockdown of C1QA in both GOhTRT high grade dysplastic cells and SK-GT-4 oesophageal adenocarcinoma cells. Cells were seeded at 5×10^3 and 7.5×10^3 cells/well for GOhTRT and SK-GT-4 cell lines respectively, and reverse transfected as per section 2.3. Briefly, cells were seeded in the 12-well plate and C1QA siRNA was transfected in using 2 μ L DharmaFECT 4 transfection reagent, to give a final siRNA concentration of 25nM. Real time PCR was performed as per section 2.10, in a 384 well plate and GAPDH was again used as an endogenous control. Results confirmed that C1QA silencing was highly significant ($p < 0.0001$) compared to non-targeting siRNA treated cells, in both the GOhTRT ($18\% \pm 2.4$ vs non-targeting) and SK-GT-4 ($41\% \pm 7.8$ vs non-targeting) cell lines (Figure 5.9). Results also indicated that C1QA is present within the SK-GT-4 cell line, representative of OAC, which was successfully inhibited using siLIF, suggesting that C1QA may be present in both BO and OAC.

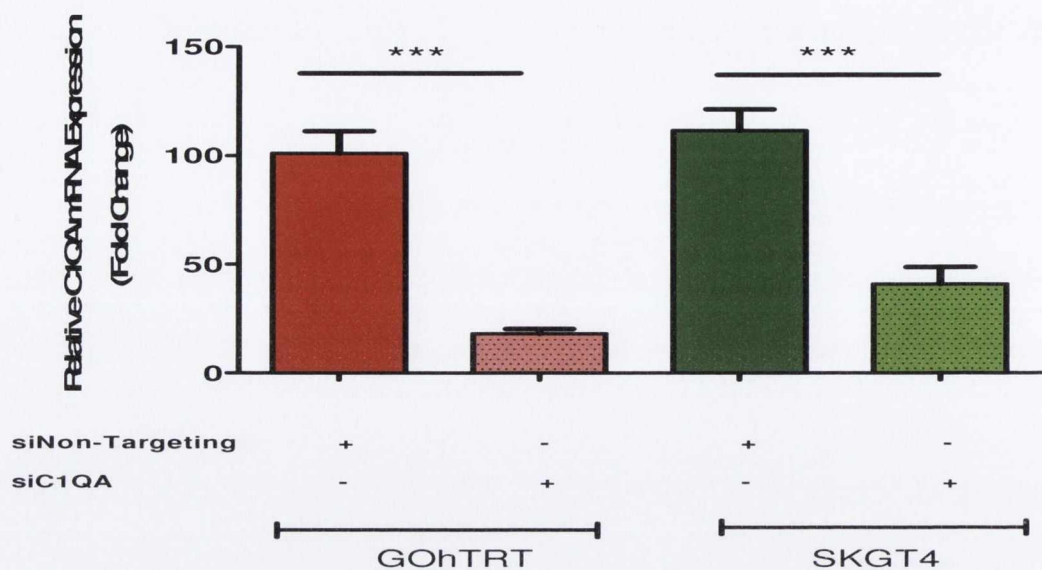


Figure 5.9: Confirmation of C1QA Knockdown at mRNA Levels. Confirmation of siRNA mediated C1QA knockdown by RT-PCR showed a reduction in C1QA expression by $82\% \pm 2.4$ in GOhTRT cells and $59\% \pm 7.8$ in the SKGT4 cells, following treatment with siC1QA compared to non-targeting negative control treatment. N=3 and results represent mean $\pm$ SEM. (t-test; ***p<0.0001)

Confirmation of C1QA knockdown was also necessary at a protein level utilising a C1QA polyclonal antibody (Abcam) in both Western blot and immunofluorescence staining. For Western blot, GOhTRT cells were seeded at 35×10^3 cells per well in a 6-well plate as per section 2.11, and each condition was setup in triplicate wells. Cells were; untreated, non-targeting siRNA treated, C1QA siRNA treated, or treated with C1 inhibitor (C1INH). For immunofluorescent staining in the GOhTRT cells, the same C1QA polyclonal antibody was used. All treatments were the same as for Western blot, except for one treatment in which cells treated with C1QA siRNA were subsequently co-treated with C1q from human serum. Cells were seeded at 5×10^3 cells/well, in a 96-well plate and siRNA reverse transfection was facilitated by use of 0.2 μ L DharmaFECT 4, for a final siRNA concentration of 25nM. At 96hrs post-

transfection, cells were fixed with 8% PFA. For immunofluorescent staining, the polyclonal C1QA antibody was used to stain for C1QA and Hoechst 33342 was used for nuclear staining. Images were taken using the GE Healthcare InCell analyser at 20x magnification.

Confirmation of siRNA mediated C1QA silencing by Western blot (Figure 5.10A), indicated a 50% knockdown compared to non-targeting siRNA treated cells after normalisation to the loading control (β -actin) per sample. C1INH is used to inhibit C1 activation of the classical complement pathway, by preventing binding of C1r and C1s, and was used as a control to show that although C1 function is inhibited, C1QA protein remains unchanged (Figure 5.10A). Immunofluorescent staining (Figure 5.10B) shows a significant decrease ($p < 0.05$) in C1QA staining between the non-targeting negative control, and siC1QA transfected cells indicating that the siRNA successfully silenced the C1QA protein.

5.3.2.2 C1q from Human Serum Increases OAC Cell Proliferation and Down-regulates C1QA mRNA Expression Suggesting a Negative Feedback Loop

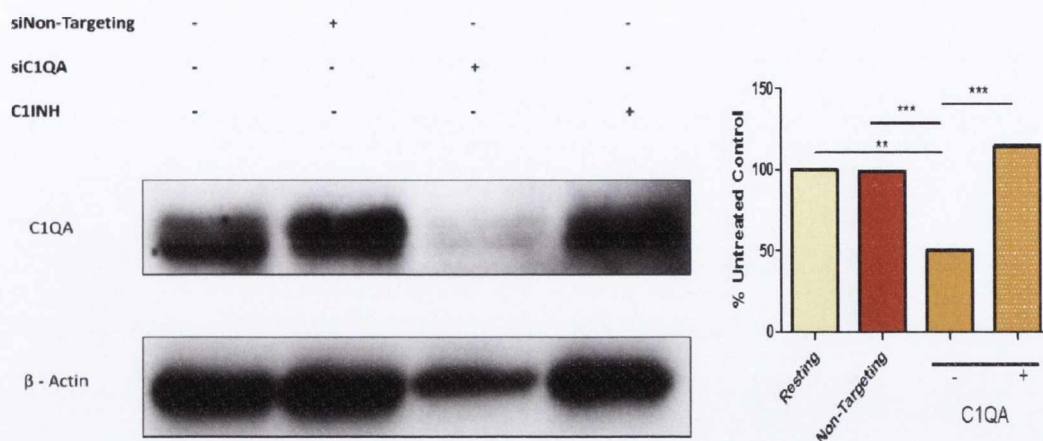
The aim of this section was to assess the effect of exogenous C1q from human serum (Sigma Aldrich), when added to cells which had undergone siRNA mediated silencing of C1QA. A concentration of 75 $\mu\text{g/mL}$ was chosen for treatment with C1q protein as it is within the recommended range, and is just higher than the level of C1q found in normal human serum (70 $\mu\text{g/mL}$). Due to the large volume of C1q needed for Western blot protein analysis, immunofluorescent staining for C1QA protein was utilised instead, which required a significantly smaller volume. Reverse transfection in the GOhTRT cells, of the non-targeting or C1QA siRNAs was facilitated by

DharmaFECT4 transfection reagent at, 0.2 μ L for MTT assay and immunofluorescence, and 2 μ L for RT-PCR, per well. At 48hours post transfection, 75 μ g/mL C1q from human serum was added to the corresponding wells. At 96 hours post-transfection, one plate was fixed and was used to detect effect on protein levels via immunofluorescence, the second plate was to detect cell viability via MTT assay, while the final plate was to detect mRNA expression via RT-PCR.

Immunofluorescent analysis of protein levels (Figure 5.10B) show C1QA protein silencing following siC1QA treatment, but upon treatment with C1q from human serum, C1QA staining was increased. This suggests either restoration of intracellular levels but alternatively this may also be due to adherence of exogenous C1q to cell surfaces. Figure 5.11A shows that siRNA mediated silencing of C1QA resulted in a significant decrease in cell viability of $28\% \pm 8$ ($p < 0.01$), while addition of C1q restored cell viability level to $95\% \pm 12$ ($p < 0.01$). Surprisingly, treatment with exogenous C1q resulted in significantly reduced levels of cellular C1QA mRNA expression of $65\% \pm 4$ ($p < 0.01$) as compared to resting non-targeting siRNA transfected cells (Figure 5.11B). Additionally, treatment of C1QA silenced cells with exogenous C1q from human serum further reduced the cellular levels of C1QA mRNA to $89\% \pm 1.5$ ($p < 0.001$) greater than siC1QA alone ($38\% \pm 4.6$) ($p < 0.01$).

A possible explanation for this surprising finding may be that, when siC1QA cells are treated with C1q from human serum, it may adhere to the cell surfaces, binding to complement specific receptors. This may then mediate a complement specific signalling pathway to reduce localised intracellular production of C1QA, thus lowering levels of C1QA mRNA, and potentially initiating a feedback loop.

A



B

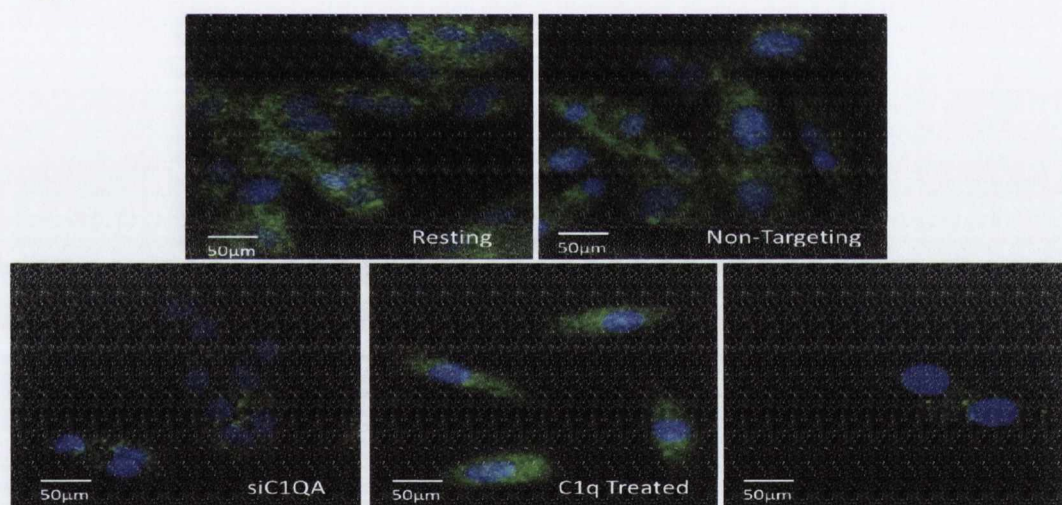


Figure 5.10: Protein Analysis Indicating C1QA Knockdown Following siRNA Treatment. siRNA mediated C1QA knockdown has been confirmed by Western blot and immunofluorescent staining. Untreated GOhTRT cells functioned as a positive control for the anti-C1QA polyclonal antibody as basal levels for the cell line has already been confirmed. A) Western blot analysis using the C1QA antibody indicated successful knockdown when treated with C1QA siRNA in which β -actin served as a loading control, B) The knockdown seen in the Western blot was also confirmed by immunofluorescent staining. Images were taken at 20x magnification. N=3. Western blot densitometry analysed using t-test (** $p < 0.01$, *** $p < 0.0001$)

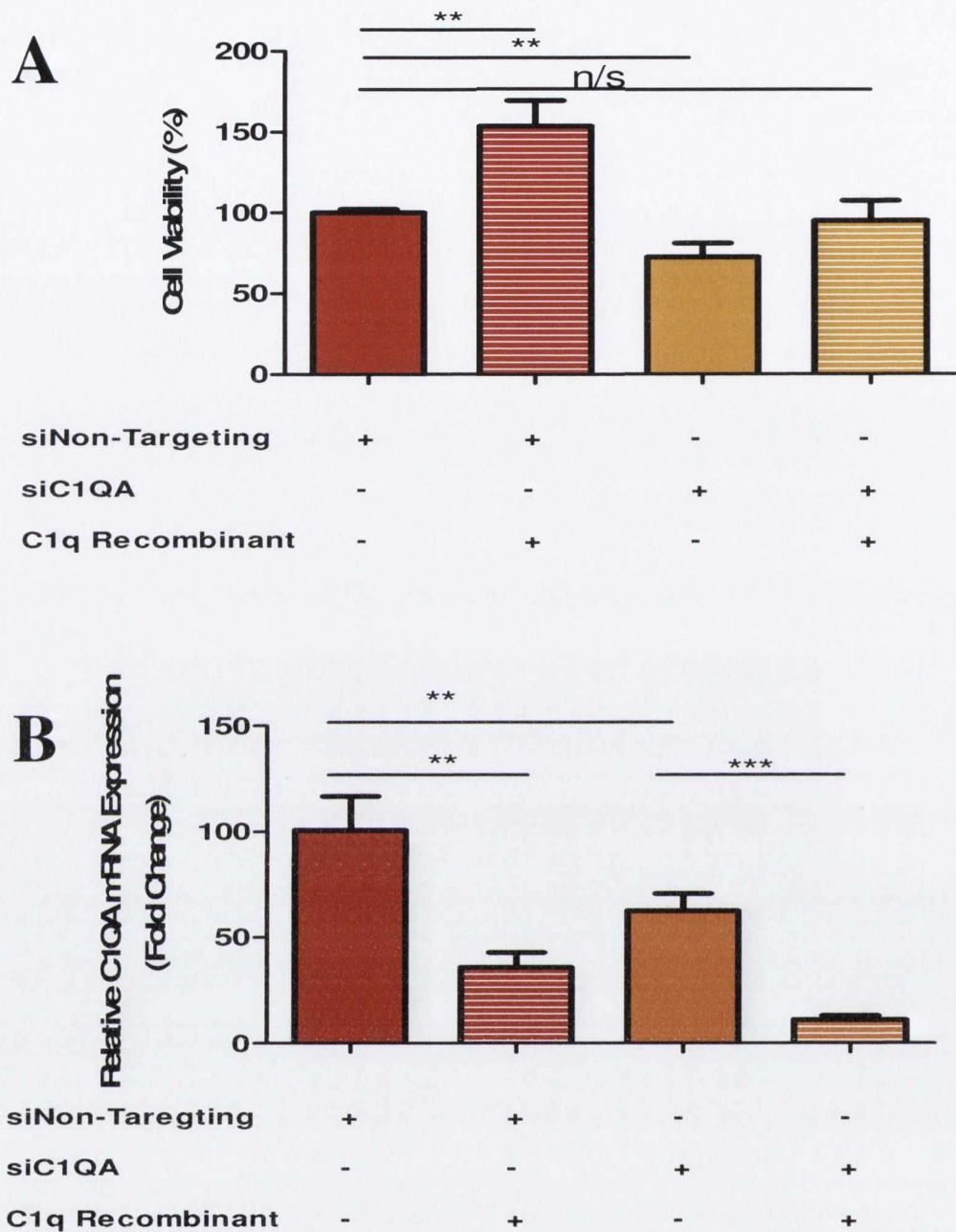


Figure 5.11: Identification of a Possible Negative Feedback Following Treatment with C1q from Human Serum. Restoration of C1QA knockdown by C1q from human serum did not increase cell viability or gene expression. A) MTT cell viability assay setup in GOhTRT cells, B) RT-PCR to quantify gene expression knockdown at mRNA levels. N=3 and results represent mean $\pm$ SEM. Results analysed using t-test (** $p < 0.01$, *** $p < 0.0001$, n/s: not significant)

5.3.2.3C1QA Silencing Increases β -catenin Stabilisation in Oesophageal Cell

Lines

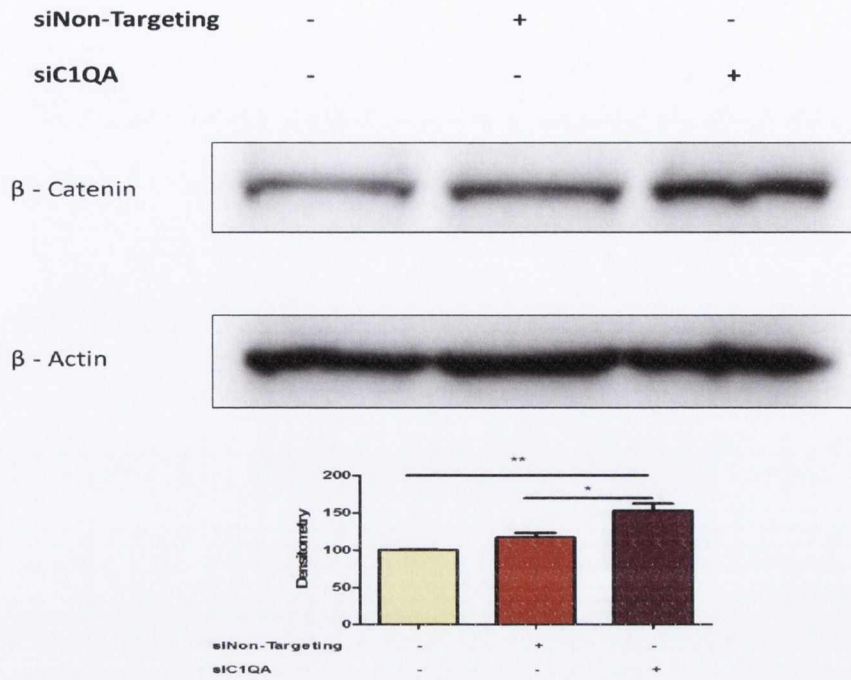
Activation of the canonical Wnt signalling pathway results in cytosolic stabilisation of β -catenin which initiates its translocation to the nucleus. Once here it has been shown to activate tumour promoting genes within BO and OAC (Clement et al., 2006a). A recent study by (Naito et al., 2012) showed that treatment of HEK293 cells with C1q from human serum stabilised cytosolic β -catenin. Thus it was postulated that the C1QA gene identified in our screen may be important within the canonical Wnt signalling pathway through stabilisation of β -catenin.

5.3.2.3.1 C1QA Knockdown Stabilises β -catenin Resulting in Translocation to the Nucleus in Oesophageal High Grade Dysplastic Cells

Cytoplasmic stabilisation of β -catenin indicates subsequent nuclear translocation (Baril et al., 2013) followed by activation of nuclear transcription. It was thus postulated that Western blots could detect any change in β -catenin status following siRNA mediated silencing of C1QA. The C1QA siRNA was reverse transfected into the GOhTRT cells and was setup as previously described. The blot was incubated with β -catenin monoclonal antibody (BD Biosciences), and anti-mouse β -actin served as a protein loading control. Immunofluorescent imaging was also carried out as previously described, using the GE Healthcare InCell analyser at 20x magnification. To generate a more detailed image of the β -catenin localisation, confocal microscopy was utilised, using 63x magnification. The GOhTRT cells were seeded at 2.5×10^3 cells/well and the C1QA siRNA was reverse transfected into the cells using 1.4 μ L DharmaFECT 4 transfection reagent per well, to give a final siRNA concentration of 25nM. The same monoclonal β -catenin antibody was used in conjunction with

Hoechst 33342 nuclear stain, and images were generated using the Zeiss 510 LSM Meta confocal microscope. Western blotting indicates a significant increase is in cytosolic β -catenin following C1QA siRNA mediated knockdown as compared to untreated or non-targeting siRNA treated cells ($p=0.005$) (Figure 5.12A). Densitometry of the triplicate experiments shows an increase in β -catenin protein expression of 53% compared to untreated cells and 36% compared to non-targeting siRNA treated cells. Immunofluorescent images (Figure 5.12B) also identifies an increase in cytoplasmic β -catenin following C1QA knockdown as compared to non-targeting ($136.075\% \pm 14$ vs $99.975\% \pm 4$) ($p<0.05$). In figure 5.13 the red arrows highlight the nuclear regions in both images showing a significant increase in nuclear β -catenin following C1QA knockdown ($p<0.05$). An increase is also noted in cytoplasmic β -catenin although this was not shown to be significant. These results demonstrate that C1QA knockdown may increase β -catenin stabilisation in high grade dysplastic oesophageal cells, through an unknown mechanism, as highlighted by the protein accumulations noted in the cytosolic fraction.

A



B

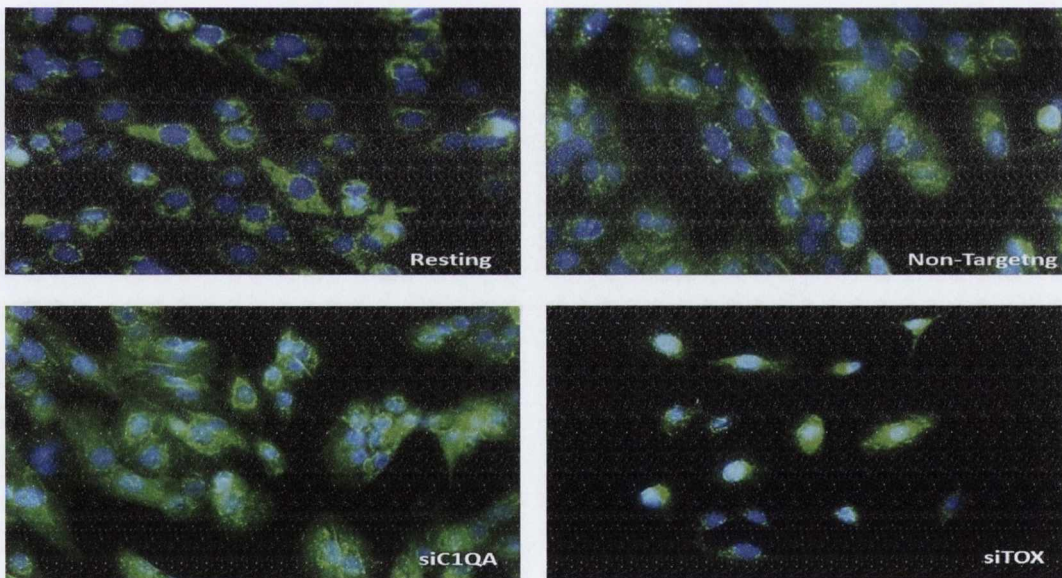


Figure 5.12: Protein Analysis of β catenin Stabilisation. A) Western blot analysis indicates an increase in β -catenin levels within the cytoplasm following siC1QA treatment. B) Immunofluorescent imaging also shows an increase in nuclear localisation of β -catenin further supporting the theory that C1QA knockdown stabilises β -catenin. Images were taken at 10x magnification. N=3. Western blot densitometry analysed using t-test (* $p < 0.05$, ** $p < 0.01$).

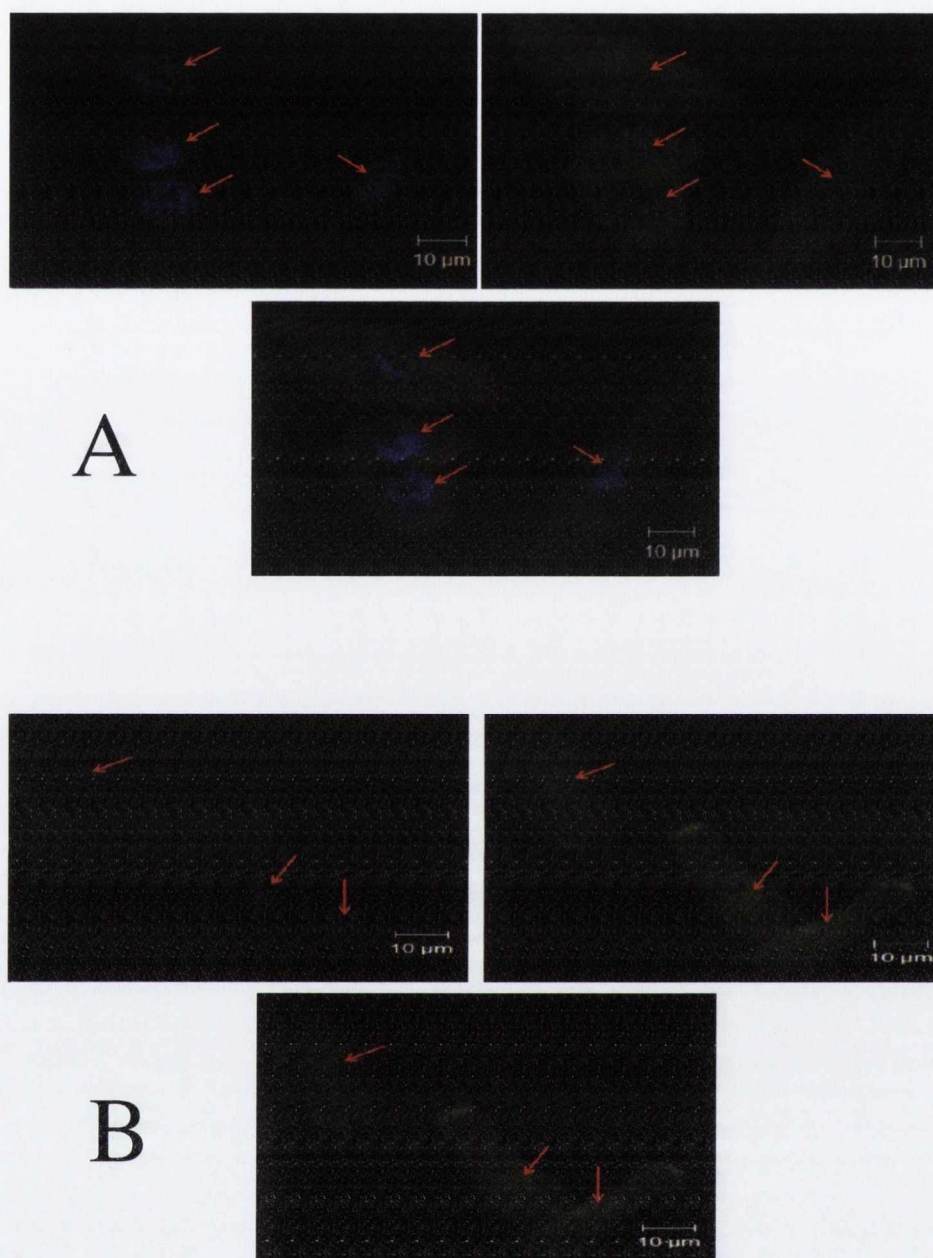


Figure 5.13: C1QA Inhibition Increases the Level of Cytosolic β -catenin in GOhTRT Cells. To further analyse changes in β -catenin status following siRNA treatment, confocal microscopy at 63x magnification identified that; A) Cells treated with non-targeting siRNA showed β -catenin staining localised to the plasma membrane, but; B) Following siRNA mediated C1QA silencing, increased cytoplasmic levels of β -catenin were seen, with nuclear accumulations (red arrows) noted. All parameters were kept constant for generation of images. N=3.

5.3.2.3.2 Luciferase Based Analysis of Wnt Signalling Induced by C1QA Inhibition

The TOPFLASH assay is a luciferase based assay which is used to measure Wnt signalling events within cells. The M50 β -catenin reporter plasmid (Super8XTOPflash) was obtained from Addgene, and contains a TCF/LEF site upstream of a luciferase reporter (Figure 2.10A). Previous research in HEK cells indicated maximal activation of this reporter is 100-fold upon Wnt activation, and up to 1,000-fold upon activation by phosphorylation mutants of β -catenin. The M51 clone is the appropriate control plasmid (Super8XFOPflash), which has a mutant TCF/LEF binding sites (Figure 2.10B) (Veeman et al., 2003).

In sequential transfections, the Hek293T cells were seeded at 5×10^3 cells per well in a 96-well plate. Cells were reverse transfected with C1QA siRNA using 0.2 μ L DharmaFECT 4, resulting in a final siRNA concentration of 25nM, as previously optimised. At 48hrs post-transfection, siRNA transfected cells were again co-transfected with the corresponding reporter plasmid; M50, M51 or empty vector pcDNA. For each sample, 20 μ L of the lysate was added to an opaque white 96-well plate, to avoid cross-talk between wells which may cause high luminescence activity. To each well, 100 μ L luciferase assay reagent II (LAR II) was added to measure firefly activity. Luciferase activity was measured using the Perkin Elmer Victor II luminometer, with a 2-second pre-measurement delay followed by a 10-second measurement period for each well as recommended by the manufacturer (Promega). Optimisation of the plasmids in Hek293T cells identified 500ng plasmid per well as the optimal concentration, when transfected with 1.5 μ L Fugene HD transfection reagent (Figure 5.14).

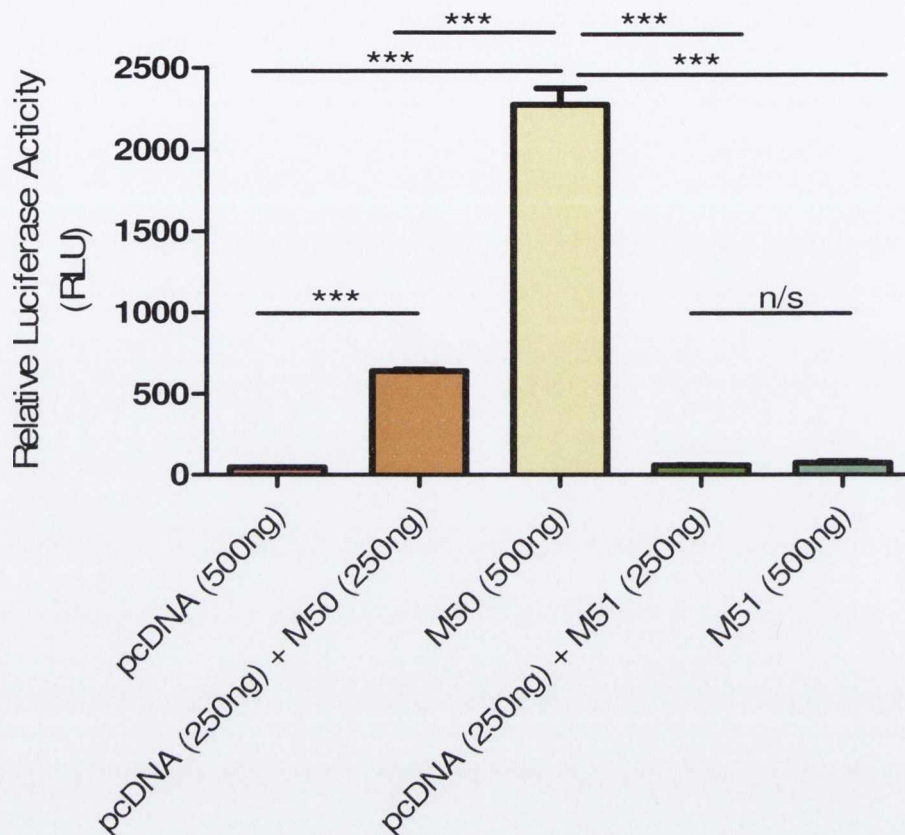


Figure 5.14: Optimisation of Luciferase Based Assay in Hek293T Cell Line. Hek293T cells were transfected with either TOPFLASH plasmid (M50) or FOPFLASH plasmid (M51), or pcDNA. Relative luciferase activity is represented by TOPFLASH/FOPFLASH readings. N=3 and results represent mean $\pm$ SEM. Results analysed using the t-test (** $p < 0.0001$, n/s: not significant).

As demonstrated in Figure 5.15, a significant increase in luciferase activity is noted upon inhibition of C1QA following siRNA mediated knockdown as compared to untreated cells ($p=0.003$) and non-targeting siRNA treated cells ($p=0.01$). These results correlate with the findings by Western blot that C1QA knockdown increases β -catenin stabilisation levels, putatively through canonical Wnt signalling as suggested by the luciferase reporter assay. Overall, this analysis has revealed that inhibition of

C1QA can activate the Wnt/ β -catenin pathway through a currently unidentified mechanism.

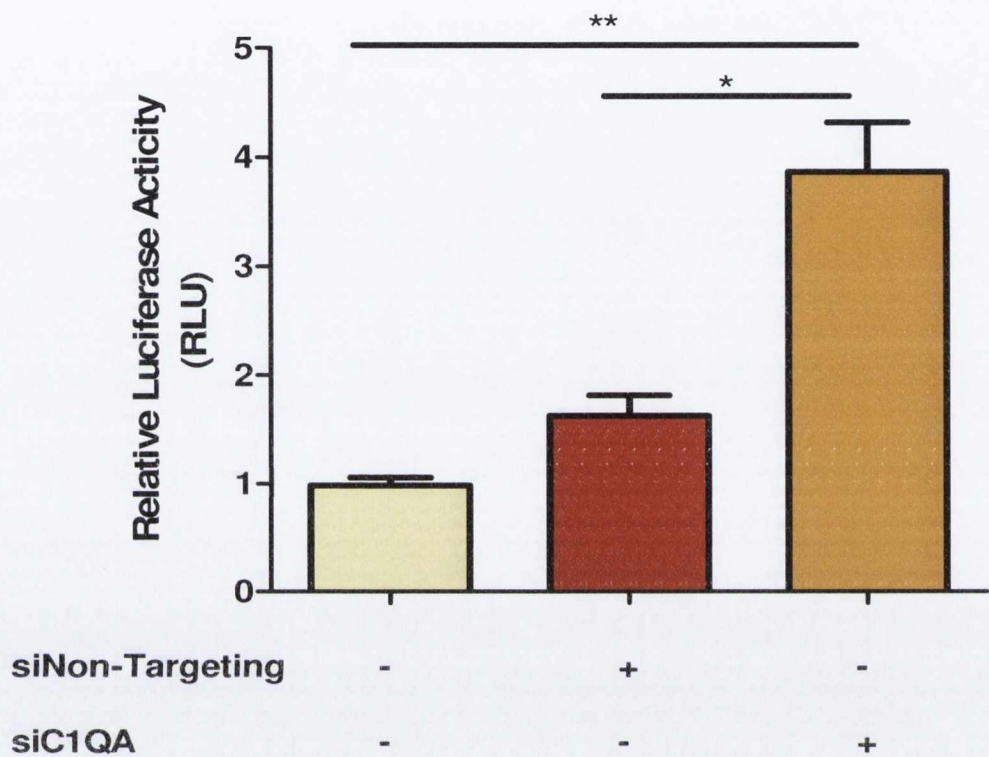


Figure 5.15: Luciferase Assay Indicates Wnt Signalling Activation Following C1QA Knockdown. Hek293T cells were sequentially transfected with either non-targeting siRNA or C1QA siRNA, followed by TOPFLASH plasmids, M50 and FOPFLASH plasmid, M51. Untreated Hek293T cells were used as a control. Relative luciferase activity is represented by TOPFLASH/FOPFLASH readings. N=3 and results represent mean $\pm$ SEM. Results analysed using the t-test (* $p < 0.05$, ** $p < 0.01$).

5.3.3 Identification of Suggested Synergistic Interactions Between a Panel of Immune Related siRNA Hits and LIF; and C1QA

Compounds which act in a synergistic manner have increased the efficacy of cancer based therapeutics but also provide evidence of pathway interactions. It was

hypothesised that inhibition of two immune genes by siRNA may decrease oesophageal cell viability further than treatment with a single individual siRNA. This may indicate possible gene-gene interactions between unrelated immune factors in their contribution to OAC cell survival. Therefore, a synthetic lethal screen was performed between LIF or C1QA and a selection of immune related genes from the druggable genome.

Table 5.1: Immune Related Genes ('Immune Panel') Used in Synergistic Analysis

MNDA	TRPV2	LIFR	TRAF4
RABGEF1	ICAM1	STAT3	HLA-G
TF	IFNW1	IL6	BMP3
KCNIP3 / CSEN	IL9R	IL6ST	CD5
MXD1 / MAD	ITGB3	C1R	IL17
RUNX2	RELA	C1S	IL11
CES1	PROK1	C1QB	IL15RA
GMPR2	TGFB1	C1QC	IL22
GSTZ1	TLR2	ILF3	IL2RA
TP53	THBS2	NFKBIL2	THBD
KIF1C	C1QA	PIP5K1B	BCL9
LIF	GPC3	ABP1	HF1
TNFRSF8	IFNA7	TGFB2	IL13
TREM2	PTCRA	BMP1	NK4

A panel of 56 immune related genes (Table 5.1) (*'immune panel'*), all of which were identified in the original druggable genome library, were arrayed in a 96-well plate. A double knockdown was performed to analyse the effect on cell viability, of this 'immune panel' combined with LIF silencing, and same then repeated for C1QA silencing. Control plates containing the 'immune panel' alone were also set up in parallel. The double knockdown was performed under the conditions already outlined. Briefly, GOhTRT cells were seeded at 10x10³ cells per well, and siRNA was reverse

transfected into the cells. Due to the use of two siRNAs, the combined final concentration of siRNA was 50nM, but the amount of DharmaFECT 4 transfection reagent remained unchanged, so that 0.2µL transfection reagent was used to transfect the siRNAs into the cells giving a final concentration of 50nM. As a control for the increase in siRNA concentration, the non-targeting siRNA (negative control) and GATA6 siRNA (positive control) were also increased to the same concentration. At 96 hours post-transfection, 3 plates were analysed using the MTT assay for cell viability.

Statistical analysis was carried out on the data generated from this experiment using R statistical software, as previously described. Quality control data indicated that the Z-factor values for all plates, were above 0.5 indicating a successful screen (Figure 5.16A&B). For hit selection, slightly less stringent cut-off values were used compared to the original druggable genome screen, although percentage cell viability remained unchanged; CV < 40%, P < 0.05, SSMD <-5, Z-Score < -1 (Table 5.2 & Table 5.3).

Table 5.2: Top 7 Genes Identified by Synergistic Analysis Between LIF and the Immune Panel

Gene Name	Raw Median	Normalised Median	Z-score Median	P-Values	SSMD	Cell Viability	100 - CV
RUNX2	0.4414	0.450914	-1.59244	0.00611	-2.26399	45.09143	54.90857
TREM2	0.4745	0.484728	-1.45673	0.008974	-2.04637	48.47278	51.52722
MAD	0.5549	0.574907	-1.17191	0.076331	-5.71587	57.49068	42.50932
BMP3	0.5806	0.601533	-1.01453	0.014276	-1.72005	60.15334	39.84666
PTCRA	0.5785	0.590969	-0.8559	0.026256	-11.3167	59.09695	40.90305
ILF3	0.6164	0.638624	-0.75133	0.047717	-10.5957	63.86241	36.13759
C1QA	0.6553	0.654425	-0.4122	0.037181	-6.01381	65.44248	34.55752

Table 5.3: RUNX2 was the Only Gene to Show an Association with C1QA from the Immune Panel

Gene Name	Raw Median	Normalised Median	Z-score Median	P-Values	SSMD	Cell Viability	100 - CV
RUNX2	0.4103	0.379345	-2.50825	0.06764	-14.765	37.93454	62.06546

As can be noted, there were no genes contained within all 4 cut-offs (Figure 5.16C&D). In the experiment with siC1QA, one gene, RUNX2 (highlighted in a red star), passed the cut-off values of; CV, SSMD and Z-Score (Figure 5.16C), while in the experiment with siLIF, 7 genes (highlighted in red stars) remained following use of the cut-off values of Z-Score, SSMD and P-value (Figure 5.16D), MAD, RUNX2, TREM2, BMP3, PTCRA, C1QA and ILF3. Overall these 7 genes were highlighted, and RUNX2 was shown to play a role in both sets of analyses. An extensive literature search of these 7 genes highlighted that 3 of these genes, RUNX2, TREM2 and PTCRA, were located within the same chromosomal area, 6p21, which had previously been highlighted as an area of polymorphic variation in BO (Su et al., 2012).

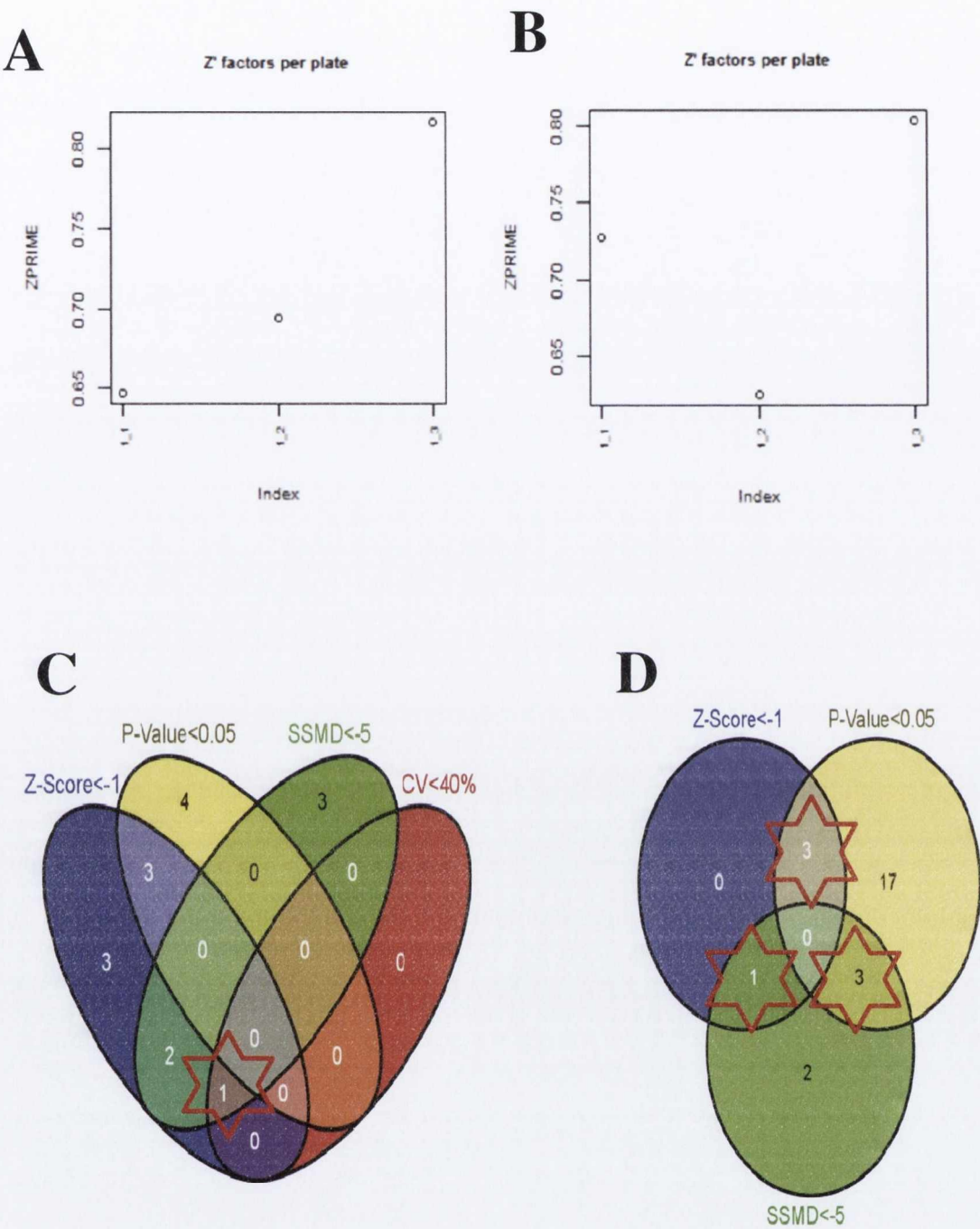


Figure 5.16: Quality Control and Hit Selection Analyses of Interactions Between the Immune Panel and C1QA and LIF. A) Z-factor values between C1QA and the immune panel were all above 0.5, B) Z-factor values between LIF and the immune panel were also all above 0.5, C) Venn diagram of cut-off results between the immune panel and C1QA, D) Venn diagram of cut-off results between LIF and the immune panel. There was no results identified yielding a CV below 40% on combination of LIF and the immune panel. N=1 and experiment was performed in triplicate wells.

5.4 Discussion

Following verification of 12 immune related genes identified as hits generated from our druggable genome siRNA screen (section 4.3.7), LIF and C1QA were identified as potential mediators of OAC cell proliferation. This body of work aimed to validate the knockdown of both genes followed by analysis to identify potential pathways through which each gene signals. The use of RNAi for identification of genes that modulate a specific phenotype, such as cell viability in a loss of function screen, can be extremely useful, but come with *caveats*. Non-specific gene inhibition is common in RNAi experiments, and can result in regulation of unintended gene(s) as well as the intended gene(s) (Maida et al., 2013). Therefore, sufficient validation of gene knockdown and use of recombinant forms of the intended protein is of upmost importance for successful identification of possible therapeutic targets.

There are three main areas in which non-specific effects can arise; lipid-mediated response, interferon response and RISC-dependent off-target effects, all of which can be eliminated with stringent siRNA design filters and sufficient optimisation of both the lipid concentration and composition (ThermoScientific, 2013). Off-target effects (OTE) are different to non-specific effects, as the former can induce quantifiable phenotype changes due to inhibition of unintended genes. Some of the major characteristics of OTEs are; target independence, sequence dependence and RISC-reliance (Birmingham et al., 2007, ThermoScientific, 2013). One study highlighting the effect of OTE's used multiple synthetic siRNAs to target MAPK14 at a low concentration (10nM) which reduced the magnitude and number of OTEs as compared to a single siRNA at the same concentration (Kittler et al., 2007). On a larger scale, a siRNA library screen of 507 kinases setup to identify HIF-1 regulators,

found significant OTE's in two out of three hits identified. These two '*hits*' directly triggered down-regulation of HIF-1 α mRNA via a 7 nucleotide motif, that was found in both the HIF-1 α mRNA and the siRNA (Lin et al., 2005). Thus, both studies concluded that pooling of siRNAs was necessary, and Lin et al. also highlighted the necessity of rescue/recovery experiments.

Given this evidence a number of methods have been developed to significantly reduce unintended gene targeting. Utilisation of bioinformatics software such as BLAST, which is used to analyse sequence specificity, can reduce the risk of OTE's. This was previously described in section 4.3.8 and the risk of this unintended gene targeting is significantly reduced due to continual removal of these types of sequences in commercially available siRNA (Sharma and Rao, 2009). Regulating the concentration of siRNA within the siRNA transfection has also been identified to substantially reduce the risk of OTE's (Birmingham et al., 2007, ThermoScientific, 2013, Persengiev et al., 2004), which can be avoided by first doing sufficient optimisation within the cells line. The use of pooled siRNA sequences has also been used to help with specificity of the knockdown, allowing for lower concentrations of the siRNA to be used. For example, Dharmacon SMARTpool technology incorporates four specific siRNA sequences which all target the same gene. Finally chemically modifying the siRNAs is also necessary to reduce the risk of off-target effects. There are different types that can target OTE's and stability, including 2'-O-alkyl modifications which are used for sense strand disabling along with stabilisation and specificity of the siRNAs. These modifications were summarised in table 3.1 in more detail. All of these principles have been identified by Dharmacon (siRNA manufacturers) and have been incorporated into their ON-TARGETplus and SMARTpool siRNA reagents. For

all validation experiments in LIF and C1QA, ON-TARGETplus siRNAs were purchased, which are guaranteed to provide potent yet highly specific gene knockdown (ThermoScientific, 2013).

The discovery that innate immune response factors such as LIF and C1QA may regulate OAC cell survival is highly significant in the oesophageal context. LIF is a secreted cytokine of the IL6 family, that, when bound to its receptor, LIFR, results in subsequent STAT3 activation which is known to regulate epithelial inflammation (Berishaj et al., 2007, Wang et al., 2013c, Silver and Hunter, 2010) (Figure 5.17). Following successful confirmation of LIF silencing at mRNA and protein levels, experiments for the restoration of LIF signalling were performed. Results indicated restoration of intracellular LIF mRNA and protein levels by the exogenous recombinant protein following siRNA mediated knockdown. ELISA immunoassay was performed on cell supernatants and confirmed that LIF silencing significantly reduced the concentration of secreted LIF. This result combined with protein analysis suggested that inhibition of LIF can decrease both intracellular and secreted LIF levels.

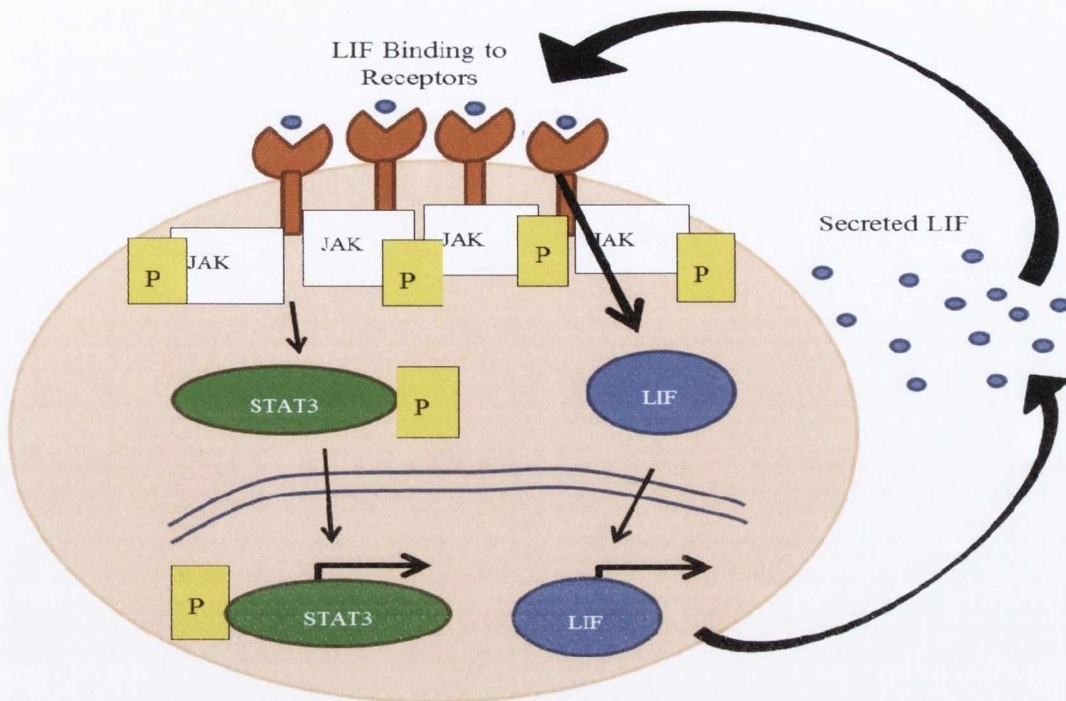


Figure 5.17: Autocrine Feedback Loop in Resting Cancer Cells. Upon LIF binding to its receptor complex, JAK/STAT phosphorylation is induced resulting in further production of LIF and STAT3 activation. LIF expression induces secretion of LIF, which in turn reactivates the feedback loop in an autocrine manner.

LIF inhibition reduces pSTAT3 phosphorylation at residue Y705, indicating that LIF may mediate STAT3 activation within oesophageal cells (Figure 5.18). Restoration of STAT3 signalling was observed upon treatment of LIF silenced cells with exogenous recombinant LIF (Figure 5.19). This indicates that the observed constitutive STAT3 activation in OAC cells may be mediated by over-production of LIF. Together with the observed increase in LIF mRNA in clinical samples, this suggests that aberrant STAT3 activation may be mediated by a dysregulated autocrine LIF-STAT3 signalling loop. Evidence suggesting that LIF may work in a paracrine/autocrine manner has previously been shown in the endometrium (Cullinan et al., 1996) but thus far has not been highlighted in oesophageal cancer. IL-6 may also act in an autocrine or paracrine manner in lung adenocarcinoma (Yeh et al., 2006). IL-1 and

TNF α have been identified as regulators of LIF in synovial fluid (Hamilton et al., 1993), which was also highlighted in articular chondrocytes and cartilage, whereby addition of these cytokines stimulated LIF production indicated again by detection of increased mRNA levels (Campbell et al., 1993).

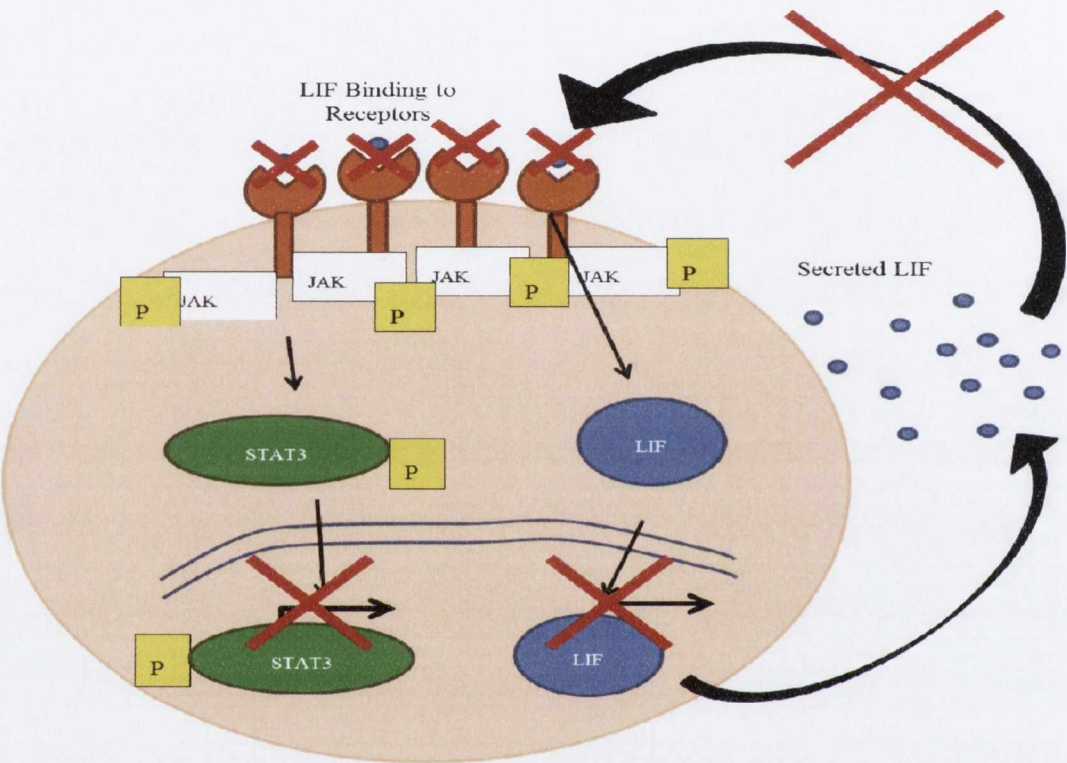


Figure 5.18: Inhibition of LIF Reduces STAT3 activation and LIF Expression. Inhibition of LIF by siRNA silences LIF and reduces STAT3 activation. Secreted LIF levels are also significantly reduced following treatment with siLIF.

In relation to oesophageal cells, inflammation caused by GORD is known to activate the NF κ B pathway which in turn induces the production of IL-1 β and TNF, which are both highly expressed in BO and OAC (O'Riordan et al., 2005). This suggests possible mechanisms through which IL-1 β and/or TNF mediated LIF over-production may result in aberrant STAT3 activation in the oesophagus, although thus far this has not been examined. Further analysis would be necessary to investigate whether both

IL-1 β and TNF status in OAC may result in alterations in LIF. LIF has also previously been implicated in the development and maintenance of Treg, which release LIF in response to antigens, and promote tumour progression (Gao et al., 2009). Tregs primarily function to prevent inflammation and maintain healthy tissue, but increased levels have been identified in OAC which is associated with poor prognosis (Ichihara et al., 2003). Further analysis has also shown that LIF can support FOXP3 expression, which can in turn coordinate the differentiation of T-lymphocytes towards Treg (Gao et al., 2009). This indicates that LIF production by OAC may result in alterations to local innate immune response inflammation, T-cell regulation and cancer survival.

STAT3 is aberrantly activated in several epithelial cancers and can also facilitate tumour promotion by creating a pro-inflammatory microenvironment (Grivennikov et al., 2009, Yadav et al., 2011, Chan et al., 2004). STAT3 can up-regulate genes that are known for their pro-survival properties such as MCL-1 and Bcl-xL, thereby inhibiting apoptosis (Hamedani et al., 2014). Binding of the members of the IL-6 family of cytokines to their receptors induces STAT3 activation and subsequent accumulation in the cytoplasm and translocation to the nucleus. For example, binding of IL-6 drives JAK/STAT3 activation which up-regulates these anti-apoptotic genes, while inhibition of STAT3 has been shown to reduce tumour growth in some cancers including pancreatic (Cardoso et al., 2012) and can initiate anti-tumour immunity within the haematopoietic system (Cardoso et al., 2012, Kortylewski et al., 2005). Several studies have also shown that inhibition of IL-6 binding to its receptor IL-6R can inhibit STAT3 activation, therefore reducing tumour growth (Chang et al., 2013, Wang et al., 2009a). IL-6 family members utilise a common gp130 receptor in

addition to member specific receptors thus, it was investigated whether LIF could modulate IL-6 levels through STAT3 activation. It is the use of this shared gp130 receptor that leads one to expect functional redundancy between the members of the IL-6 family of cytokines, whereby the inhibition of one of the genes will not affect the biological phenotype due to parallel signalling events mediated by other family members (Nowak et al., 1997). Redundancy has been previously identified between LIF and Oncostatin M (OSM), another IL-6 family member (Gearing et al., 1992), both of which are closely located on chromosome 22q12.2. Additionally, human OSM can signal using heterodimerisation of gp130 with either the LIF receptor (LIFR) or OSM receptor (OSMR), known as the LIF/OSM receptor (Knight et al., 2003, Thoma et al., 1994). Given this, it was postulated that genetic redundancy may occur with respect to LIF and IL-6, and that LIF knockdown should not affect IL-6 gene expression levels. An RT-PCR experiment was setup to analyse the effect of LIF knockdown, and subsequent restoration with both LIF and IL-6 exogenous recombinant proteins, on IL-6 levels. Results showed that siRNA mediated LIF knockdown significantly reduced IL-6 levels as compared to cells treated with non-targeting siRNA. Addition of exogenous IL-6 recombinant increased IL-6 mRNA gene expression indicating functionally an operational STAT3 pathway. Significantly increased levels of IL6 gene expression were also induced following treatment of recombinant LIF. These experiments suggest that redundancy is not present between IL-6 and LIF and that LIF is the predominant factor regulating both IL6 levels and STAT3 activation status in these cells. Non-redundant characteristics within the IL-6 family of cytokines have previously been demonstrated. For example, as mentioned OSM utilises the gp130 and LIFR receptors, but OSM can also bind to its own receptor, OSMR, indicating a non-redundant function within the IL-6/STAT3

signalling pathway (Auguste et al., 1997). Both IL-6 and LIF are necessary for the inflammatory response to brain injury, but a double knockout of these two genes does not result in a more pronounced phenotype as compared to individual knockouts (Sugiura et al., 2000). A follow-up study by the same group found that rather than acting in a redundant fashion as expected, that IL-6 levels following CFA-induction were significantly decreased in LIF KO mice as compared to wild-type (WT) mice ($p<0.03$) (Zhu et al., 2001). It was thus concluded that IL-6 and LIF must act in series rather than in parallel, with one gene controlling the other. Following from these initial studies, the treatment of Schwann cells with IL-6 was shown to induce LIF and again identified that double knockout of LIF and IL-6 did not induce an increase in phenotype (Tofaris et al., 2002). Our findings indicate that LIF may regulate IL-6 expression, indicating that IL-6 mediated STAT3 activation may result in production of more LIF (Figure 5.19). This may be another possible mechanism through which LIF may be over-produced. Chronic inflammation can lead to release of IL-6, a pro-inflammatory cytokine, which can promote tumourigenesis through activation of the STAT3 signalling pathway (Becker et al., 2004, Berishaj et al., 2007, Bollrath et al., 2009). Thus it may be postulated that inhibition of LIF regulated IL-6 could reduce the inflammatory infiltrate associate with OAC, by decreasing IL-6 levels.

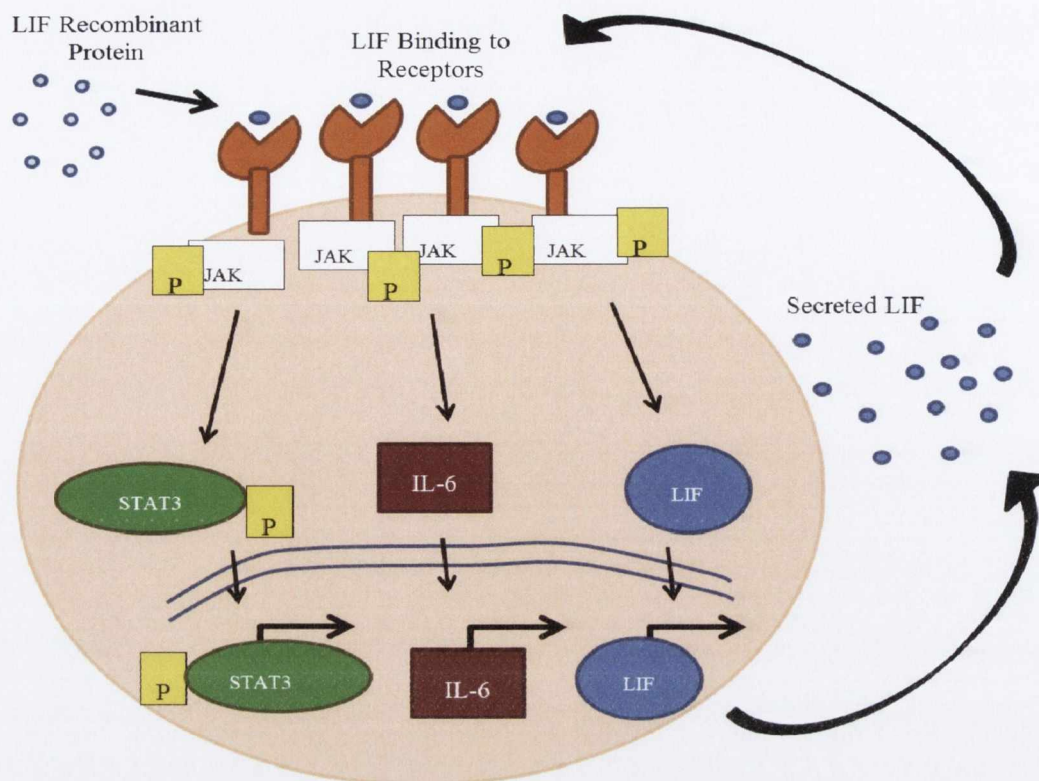


Figure 5.19: Addition of Recombinant LIF Protein so siLIF Treated Cells can Restore STAT3 Signalling and IL-6 Production. Following siRNA mediated LIF silencing, treatment of exogenous recombinant LIF can restore both STAT3 signalling and IL-6 expression. This indicates redundancy does not occur between LIF and IL-6, and also that LIF may regulate IL-6.

In parallel analyses, the role of complement component C1QA in OAC cell survival was a subject of this thesis. The complement system plays a key role in the innate immune response by identification and subsequent destruction of invading organisms, and it can also control immunological and inflammatory processes. It is thus hypothesised that, since complement recognises diseased or damaged cells, it should identify malignant cells and destroy them before a tumour develops. Research has shown that cancer cells avoid recognition and targeting by the complement system by either expressing membrane bound complement regulatory proteins (mCRPs) or secreting complement inhibitors (Donin et al., 2003, Jurianz et al., 1999). C1QA,

C1QB and C1QC genes/proteins are combined to form the heterotrimer protein C1q; the subcomponent most widely studied for its role in disease, rather than single C1q components individually i.e. C1QA. C1q then binds to the cell surface receptors, inducing activation and secretion of the subcomponents, including C1QA, which initiate a C1QA autocrine loop (Figure 5.20).

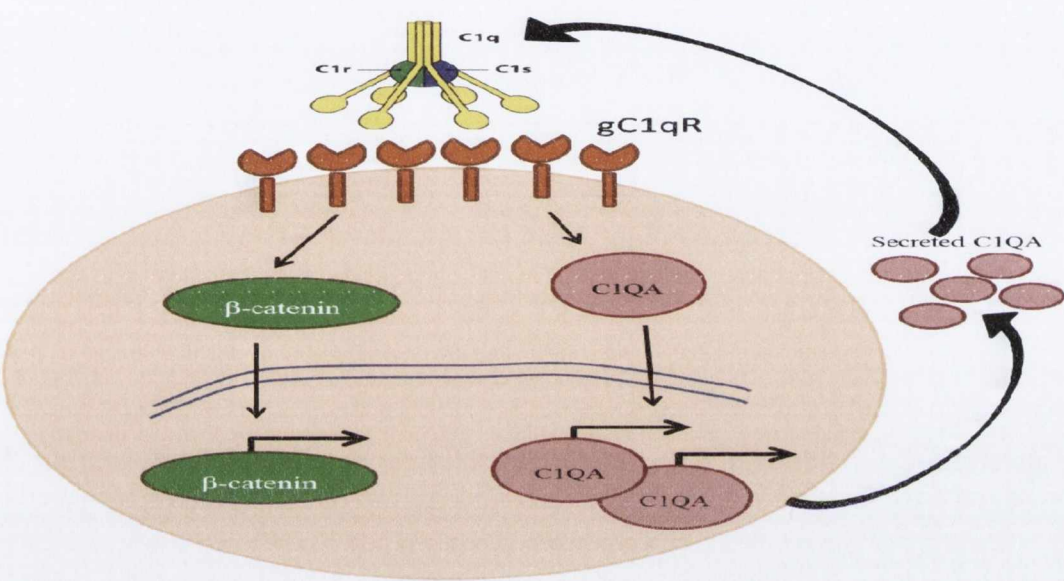


Figure 5.20: Normal Activation and Secretion of C1QA and β -catenin. C1QA binds to C1QB and C1QC to form C1q, which then forms C1 by binding to C1r and C1s. The globular heads of C1 then bind to the cell surface receptors to induce C1QA and β -catenin activation, resulting in secretion of C1QA initiated an autocrine feedback loop.

Addition of C1q from human serum to negative control cells increased cell viability significantly ($p<0.05$) as compared to untreated negative control cells. This indicated that C1q may act a growth factor in oesophageal cells which is in contrast to suggestions that complement activation reduces tumour cell viability. For example, complement activation via increased C1q deposition, can result in epidermoid carcinoma cell lysis upon EGFR inhibition, using a combination of cetuximab and matuzumab (Dechant et al., 2008).

This thesis has outlined that inhibition of C1QA via siRNA reduces intracellular protein levels of C1QA (Figure 5.21), but immunofluorescent imaging investigated the combination of both siC1QA and C1INH. Results showed a reduction in C1QA immunofluorescent staining, which was further decreased upon addition of C1INH. This suggests that inhibition of the whole first complement component, C1, can significantly reduce C1QA levels in oesophageal cells. Thus, determination of the effect siC1QA and C1INH combined treatment would have on cell viability would be of particular interest.

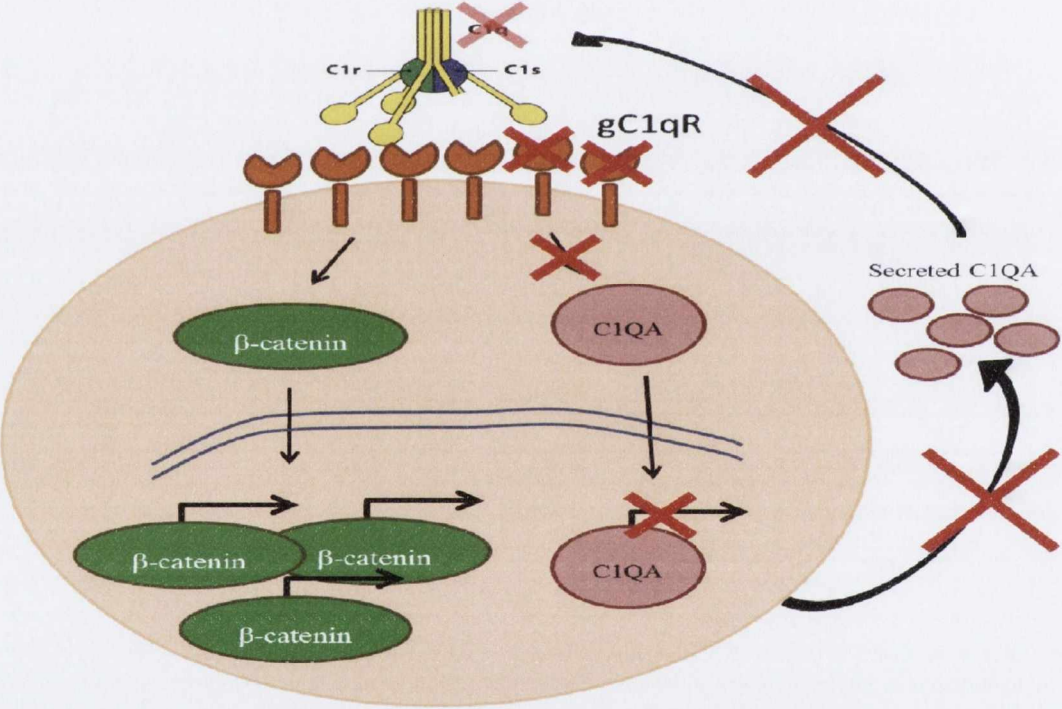


Figure 5.21: Inhibition of C1QA Can Decrease Intracellular and Secreted Levels of C1QA, While Increasing β -catenin Activation. Following siRNA mediated inhibition of C1QA, the protein is no longer produced and thus is not secreted. Thus, C1QA cannot bind to the cell surface receptors halting further production. C1QA inhibition also appears to alter β -catenin activation, which appears to be increased as compared to cells not treated with the C1QA siRNA.

A recent study with regard to aging cells, identified that addition of C1q from human serum to Hek293 cells can increase the level of canonical Wnt signalling by β -catenin stabilisation (Naito et al., 2012). Wnt signalling is either transduced by the canonical Wnt signalling pathway which is β -catenin dependent or through the non-canonical Wnt signalling pathway which is β -catenin independent. Activation of the canonical Wnt signalling pathway has been shown to induce cytosolic accumulations of β -catenin which can then enter the nucleus to bind to TCF/LEF which induces the activation of target genes including PTTG, Wnt3a and RUNX2 (Zhou et al., 2005, Zhang et al., 2009b, Dong et al., 2006). Within the BO/OAC setting, β -catenin accumulations in the cytoplasm and subsequent translocation to the nucleus have been identified in the metaplastic progression to low- and high-grade dysplasia, and BO (Clement et al., 2006a, Moyes et al., 2012). It was thus postulated that C1QA silencing may result in canonical Wnt signalling pathway alterations. As can be seen in figure 5.12, Western blot analysis indicated a significant increase in cytosolic β -catenin upon C1QA knockout ($p=0.005$). This was confirmed by immunofluorescent staining utilising the InCell and confocal microscopes, which highlighted nuclear accumulations of β -catenin. To confirm that this increase is due to activation of the canonical Wnt pathway, the Wnt luciferase assay was utilised. This assay has been developed specifically for detection of Wnt signalling using a plasmid containing TCF/LEF sites upstream of a luciferase reporter (Veeman et al., 2003). Sequential transfection of the C1QA siRNA and luciferase plasmids yielded a significant increase in luciferase activity as compared to non-targeting siRNA or untreated cells (Figure 5.15). From these findings it is suggested that C1QA knockdown can reduce cell viability which may be induced by Wnt signalling (Figure 5.21). The canonical Wnt pathway is activated by the binding of Wnt ligands to Frizzled and LRP6,

leading to phosphorylation of LRP6 and which dissociates β -catenin from Dishevelled resulting in cytosolic β -catenin accumulations. Results generated from the luciferase assay based TOPFLASH assay then suggest that C1QA may signal in a similar manner to Wnt ligands, inducing β -catenin activation.

Wnt signalling due to nuclear translocation of β -catenin has been implicated in the development and progression of several cancer types (Clement et al., 2006a, Fragoso et al., 2012, Reed et al., 2008, Uematsu et al., 2003), but increases in β -catenin levels leading to decreased cell proliferation of tumours has only been identified in multiple myeloma and malignant melanoma (Chien et al., 2009, Edwards et al., 2008). Multiple myeloma develops within the cell plasma, within the bone marrow and, while Wnt signalling is known to play a key role in the regulation of bone mass, it is also linked to the development of multiple myeloma (Oshima et al., 2005). Increased levels of Wnt signalling within multiple myeloma, can reduce tumour burden by increasing both osteoblast levels and bone formation in conjunction with reducing the number of osteoclasts in a murine model (Edwards et al., 2008). Supporting this, activation of Wnt signalling may also result in increased β -catenin but no observable proliferative effects in B-cell neoplasia (Qiang et al., 2003). The caveat associated with these studies is that although *in vitro* cell proliferation may not be increased, *in vivo*, these effects may depend on the microenvironment itself. Wnt activation has also been shown to decrease proliferation of melanoma cells, as demonstrated in both cells, for staining of the proliferation marker Ki-67, and tumour grafts, which correlates with increased patient survival (Chien et al., 2009, Bachmann et al., 2005). Although there are few studies indicating that increased canonical Wnt activation can reduce cell proliferation, as demonstrated it is not an entirely new concept.

Immunohistochemical staining of oesophageal tissue for C1QA has not previously been published, although staining for globular heads of C1q receptor (gC1qR) (section 1.5.3), has shown high levels in OAC as compared to normal squamous oesophageal tissue (Rubinstein et al., 2004). This finding, in combination with the findings of this thesis suggest that addition of C1q from human serum could bind to the cell surface receptors (gC1qR), thus signalling in an autocrine manner to halt production of more C1q subcomponents, including C1QA. This may account for the significant reduction in C1QA mRNA levels found upon treatment with both siC1QA and C1q from human serum (Figure 5.22).

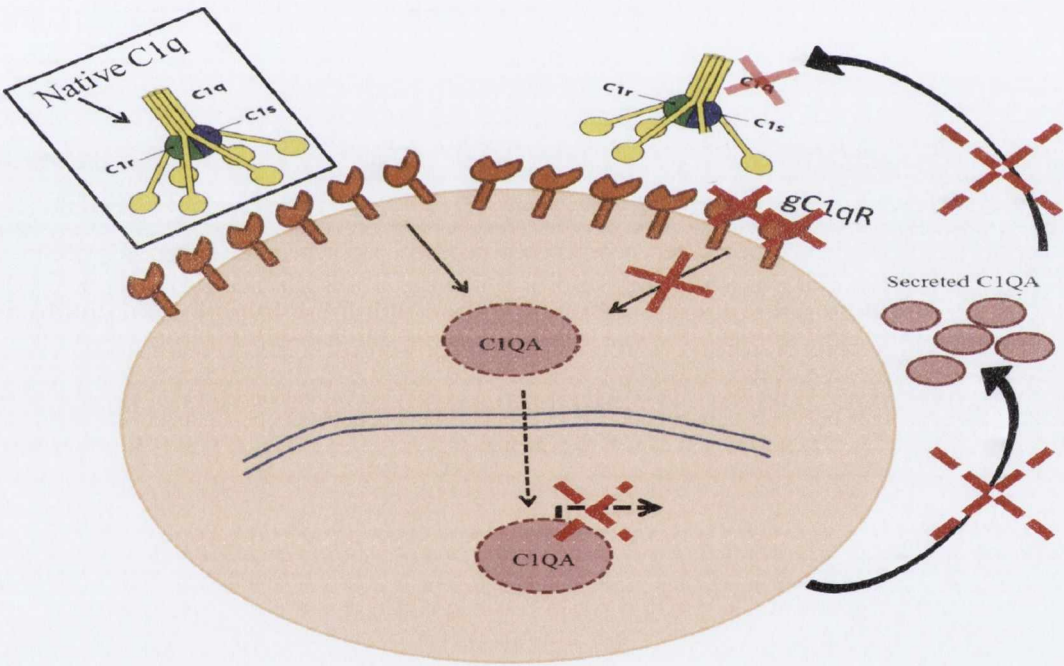


Figure 5.22: Addition of Native C1q may Halt Further Production of Intracellular C1QA. Following siRNA mediated inhibition of C1QA, activation and secretion of C1QA is inhibited, but upon addition of native C1q, a further intracellular decrease in C1QA levels is noted. This may be attributed to C1q binding to the cell surface receptor gC1qR which suggests signalling in an autocrine manner, thus halting production of more C1q subcomponents.

The discovery of a significant number of immune related genes potentially regulating OAC cell survival from the siRNA screen suggests that there may be a direct link between inflammatory factors and oesophageal carcinogenesis. In relation to the immune factors LIF and C1QA, it was investigated whether synergistic responses in cell viability could be achieved through combined inhibition of either C1QA or LIF and additional immune factors performed in a matrix fashion. This immune panel was created using immune related genes from the druggable genome library used for the original screen. Evidence for synergy between the IL-6 family of cytokines and other genes has previously been shown. LIF and BMP2 act in synergy on primary cardiomyocytes to protect against Doxorubicin-induced injury (Izumi et al., 2006), while it was also shown that BMP7 as well as BMP2 induce astrocyte differentiation in synergy with IL-6 family of cytokine including LIF or IL-6 (Yanagisawa et al., 2001). Although little evidence has been shown for synergistic interactions with complement component, one paper has shown that complement can promote the development of T-helper 17 cells via synergistic interaction with TLR signalling and IL-6 production (Fang et al., 2009).

Seven genes were highlighted as having a possible synergistic effect with LIF, which include MAD, RUNX2, TREM2, BMP3, PTCRA, C1QA, IL-9R and ILF3. C1QA has already been highlighted throughout this body of work, but no known synergy exists between LIF and C1QA to date, although as previously outlined, there appears to be a synergistic interaction between complement and IL-6. Therefore this warrants further investigation. MAD (MXD1) encodes a member of the MYC family of transcription factors which are known to mediate cell proliferation, differentiation and apoptosis for example the oncogenic transcription factor, c-MYC, is associated with

aggressive cancers, and is overexpression in BO (Schmidt et al.). A recent study has confirmed that c-MYC and MAD are overexpressed in BO (Boult et al., 2008). No evidence has been published of synergy between LIF and MAD or MYC family proteins, but this interaction may be of particular interest to analyse how LIF knockdown would affect MAD levels within BO/OAC cells. As previously shown, a synergistic interaction has been highlighted between LIF and both BMP-2 and BMP-7. Within CRC, silencing of BMP-3 is necessary in the early stage (Loh et al.), while polymorphisms within BMP-3 have been associated with onset of papillary thyroid cancer (Kim et al., 2013b). BMP-3 is the most abundant BMP protein within the bone, but it has not been shown to have a synergistic interaction with LIF, or any other gene. ILF3 has been identified as a LRH-1/NR5A2 binding protein which is a transcription factor that regulates target genes known to be involved in bile acid homeostasis including CYP7A1 and CYP8B1 (Lee et al., 2008). ILF3 can synergistically interact with PRMT1 and PGC-1 α , to promote LRH-1 gene expression (Ohno et al., 2011), but this is the only publication indicating synergy with ILF3, although its role in LRH-1 expression and subsequent bile acid production may be of particular interest in reflux induced cancer promotion in BO and OAC.

Of the seven genes identified, three genes were found to be located within the same chromosomal region on 6p21; PTCRA, TREM2 and RUNX2. The triggering receptor expressed on myeloid cells 2 (TREM2) protein is made in myeloid cells within the bone marrow. It interacts with TYROBP protein on the cell surface, where they form a complex which can activate immune cells such as dendritic cells triggering the inflammatory response. Within disease, a genetic variant within the TREM-2 gene, p.R47H, has been shown to increase Alzheimer's disease (AD) risk. It has also been

highlighted that within lung cancer patients TREM-2 may act as a negative immune regulator which can promote lung cancer progress (Yinan et al., 2013). Pre T-Cell antigen receptor alpha (PTCRA), is only found in immature T-cells and can modulate regulation in early T-cell development. Recently PTCRA has shown potential as a *Notch* regulator, which is a pathway known to be involved in both development and progression of several cancers including breast, prostate and oesophageal cancers (Al-Hussaini et al., 2011, Zhu et al., 2013a, Clemons et al., 2013). There have been no publications thus far highlighting synergistic interactions between TREM-2 and PTCRA with any other gene, nor has there been any publications linking either gene with the IL-6 family of cytokines, or STAT3. Therefore, if synergy does exist between LIF and TREM2 and PTCRA, then it would be the first reported link. One gene in particular, RUNX2, showed significant synergy with both LIF and C1QA and is therefore of particular interest for further research. RUNX2 is a member of the RUNX family of transcription factors which share a common Runt domain involved in DNA-binding and transactivation. It acts as a scaffold for nucleic acids and regulatory factors involved in skeletal gene expression and it has been shown that increased RUNX2 expression is found in several human malignancies including prostate, pancreatic, thyroid and breast cancers (Tandon et al., 2014, Niu et al., 2012, van der Deen et al., 2010, Kayed et al., 2007). The fact that these 3 genes are all located within chromosomal region 6p21 is of particular interest, as variants within this region have been highlighted as being associated with BO, in which an Irish cohort was utilised (Su et al., 2012). The 'major histocompatibility complex' (MHC) region, as previously indicated, is located within the 6p21 region and contains genes which are known to be involved in the immune response and that are involved in antigen recognition. A more recent study has shown that variations within the MHC

region could confer risk of OSCC within a Northern Chinese population (Shen et al., 2014). SNPs were identified within HLA-DQA1 and TRIM27 genes which conferred risk, and immunohistochemical (IHC) staining was shown to be significantly higher ($p < 0.001$) in OSCC tissue as compared to normal oesophageal tissue (HLA-DQA1: 69.4% vs. 26.8% and TRIM27: 77.6% vs. 47.8%) (Shen et al., 2014). Therefore the finding that 3 of the genes identified within our *immune panel* screen of oesophageal cells are located within this region may be of particular interest and warrants further investigation in relation to their role in the immune response, and the link to BO and OAC. This work is currently being undertaken by another member of the team.

Overall, this body of work has highlighted the role of two immune related genes, LIF and C1QA, in oesophageal cell proliferation. LIF has been identified as a key regulator of STAT3 activation and it is suggested that LIF may also act in series with IL-6 rather than in parallel. Along with LIF, C1QA has been identified as a novel regulator of BO, acting through the canonical Wnt signalling pathway. Interestingly it has been shown that C1QA knockdown increases Wnt signalling and β -catenin stabilisation, resulting in a reduction in cell proliferation. This finding is novel in relation to BO, as it has only been shown to occur in multiple myeloma and malignant melanoma previously. Further to this, an association has been found between both LIF and C1QA, and a panel of 56 other immune related genes. Of particular interest is the association between both genes and RUNX2, while a significant increase in RUNX2 may also contribute to tumourigenesis in OAC. These novel findings may prove of great interest and benefit to OAC patients, but they will require further investigation in a wider panel of oesophageal cells, clinical interpretation and testing in suitable cancer models.

Chapter 6

General Discussion

6 Discussion

Barrett's oesophagus (BO) is a pre-malignant condition which is associated with gastroesophageal reflux disease (GORD) and is a known precursor to oesophageal adenocarcinoma (OAC) (Su et al., 2012). The incidence of oesophageal cancer is on the increase; therefore developing new methods of treatment is key to improving the quality of life for patients, and overall survival. If BO is diagnosed early, treatments can include proton-pump inhibitors (PPI) which can reduce the amount of gastric acid secretion and laparoscopic fundoplication as symptomatic treatment for underlying reflux, or for more advanced BO, surgical options such as endoscopic mucosal resection (EMR), radio-frequency ablation (RFA) or photodynamic therapy (PDT) may be performed.

For patients diagnosed with advanced OAC, treatment options are limited to chemoradiotherapy and surgery, with many tumours unresectable. Therefore the development of new therapeutic approaches aiming to downstage the tumour to a resectable size could result in more effective and less invasive treatment approaches. With this in mind, the utilisation of RNAi technology has shown great potential for the identification of targets for the treatment of diseases, through identification of gene function using both loss-of-function and synthetic-lethal analyses. The use of siRNA libraries, containing thousands of siRNAs, has contributed greatly to the understanding of the role many genes play within specific pathways, and how they react upon drug treatment. The main aim of this body of work was to identify novel therapeutic targets for OAC through the development of a siRNA screen measuring OAC cell survival.

The success of RNAi library screens at identifying genes involved in cancer has been highlighted in several studies, varying in design, from *in vivo* screens as highlighted in breast cancer (Iorns et al., 2012), to synthetic lethal screens performed in pancreatic and metastatic myeloma for example (Fredebohm et al., 2013, Qin et al., 2013a) and loss-of-function screens such as in multiple myeloma (Lamy et al., 2013). Some targets defined by these technologies have been progressed to validation in clinical specimens such as targeting PTEN resistance to trastuzumab in breast cancer (Berns et al., 2007). Many of these screens used shRNA (Lamy et al., 2013, Fredebohm et al., 2013, Iorns et al., 2012) which are beneficial for longer gene silencing as compared to siRNA which were used in our screen, but they require a more complex transfection, utilising plasmids, than siRNA. The screen performed in this thesis was a loss-of-function screen in adherent cells examining the resultant effects on cell viability.

Most siRNA screening studies use the t-test and post-hoc Mann-Whitney or similar metrics, most commonly the Z-factor, for assessment of quality. For example, two quality metrics, the Z-factor and SSMD, were utilised following the development of an automated method for image analysis (Talati et al.). Similarly, a RNAi screen identifying TNK1 in the growth and survival of pancreatic cells used the reduction in cell viability as compared to a negative control, as an indication of transfection success (Henderson et al., 2011). This indicates a lack of sufficient quality control metrics simply relying on a decrease in cell viability. This use of minimal statistical analysis is also highlighted with respect to selection of hits in several studies including use of just the robust Z-score (Panda et al., 2013), or a decrease in cell survival (Goel et al., 2014). Statistical quality analysis within this thesis utilised; t-

test, Z-factor and SSMD to confirm successful screen implementation with high confidence. Subsequent statistical analysis performed for identification of hits within this thesis combined the results of four distinct metrics; t-statistic, robust Z-score, SSMD and percentage cell viability, to identify siRNAs generating results below pre-set cut-off values, thus identifying siRNAs that reduce cell viability significantly. Using multiple statistical tests to determine the screen quality and identify siRNAs that decrease cell survival significantly, allows for confidence that the genes identified should not be affected by off-target effects.

The siRNA library utilised in this thesis targeted genes within the human genome that express proteins which are targetable by drug-like molecules (Hopkins and Groom, 2002). Quality control analysis of the data obtained from the screen confirmed a successful assay in terms of quality and statistical analysis for hit selection using stringent cut-offs identified 118 high confidence hits which fulfilled significance tests in four separate analyses. Pathway and gene ontology analysis was conducted on this panel, resulting in identification of 12 immune related genes found to significantly reduce GOhTRT cell viability, which were subsequently verified in follow-up experimentation. From this panel two genes in particular, complement component 1, subcomponent Q, A chain (C1QA) and Leukaemia Inhibitory Factor (LIF), were identified for functional validation in this thesis, however others have been verified by different investigators within the group.

Leukaemia Inhibitory Factor (LIF) is a secreted cytokine of the IL-6 family that is involved in development, infection and inflammation. As such, LIF also shares a receptor, gp130, with IL-6, which signals through the activation of JAK kinases,

leading to STAT3 phosphorylation (pSTAT3) (Berishaj et al., 2007). STAT3 activation is known to induce inflammation (Wang et al., 2013c, Silver and Hunter, 2010), and can induce the expression of growth and angiogenic factors, as well as other cytokines. Constitutive activation of STAT3, as noted in a number of malignancies (Berishaj et al., 2007, Bollrath et al., 2009, Dvorak et al., 2007), is thought to promote and sustain tumour formation and localised inflammation. Additionally, STAT3 may also suppress anti-tumour immunity as demonstrated by IL-6 mediated up-regulation of Bcl-2 and Bcl-xL following IL-17 exposure (Wang et al., 2009c). Oesophageal tissues have also been analysed for STAT3 activation, with increasing pSTAT3 IHC staining shown from normal oesophageal squamous epithelium through the oesophageal carcinogenic sequence to OAC (Zhang et al., 2011). Functional validation in this project indicated that siRNA mediated silencing of LIF reduced the observed constitutive STAT3 activation in addition to effects on GOhTRT cell viability. Furthermore, treatment of oesophageal cells with exogenous recombinant LIF also demonstrated LIF mediated STAT3 activation suggesting a mechanism for autocrine activation which has been observed in IL-6 signalling in cancer (Yeh et al., 2006). Further work also highlighted that LIF and IL-6 may work in series rather than in parallel as previously hypothesised, by demonstrating that inhibition of LIF using siRNA significantly reduced IL-6 mRNA. Overall, these findings suggest that LIF is a driver of STAT3 activation, which can have a major impact on IL-6 levels as a change in LIF levels can directly impact in IL-6 mRNA. This suggests that inhibition of LIF in clinical tissues could reduce levels of IL-6 and subsequently STAT3 activation, thus mediating tumour-promoting inflammation, while also decreasing tumour size by reducing cell proliferation. Binding of IL-6 family cytokines can induce phosphorylation of JAK, STAT3 and mitogen-activated

protein kinase (MAPK) in cardiac myocytes (Kunisada et al., 1996) and carcinogenesis (Chang et al., 2013). Thus, upon binding of LIF to its receptors (gp130/LIFR), it is hypothesised that this may result in Janus Kinase (JAK) phosphorylation resulting in subsequent STAT3 activation in OAC. Therefore, an examination of the kinome could be used to investigate if the mechanism underlying the observed reduction in STAT3 activation, following siLIF treatment, may be by inhibition of JAK/STAT phosphorylation.

C1QA is a constituent of the C1q subcomponent of the complement system whose deficiency has been shown to be associated with forms of *Lupus erythematosus*, a long-term autoimmune disorder. Results outlined in this thesis indicate that addition of C1q can act as a growth factor to increase resting oesophageal cell viability as compared to untreated cells, while also reducing the level of intracellular C1QA through saturation of cell surface complement receptors, thus indicating a functional feedback loop. Thus far, there is no known link between C1QA and either BO or OAC. In aging cells, C1q has been observed to bind to Frizzled receptors thus inducing C1s-dependent cleavage of LRP6, and subsequent Wnt signalling activation (Figure 6.1) (Naito et al., 2012). Additionally, human serum can activate canonical Wnt signalling which correlates to abundant C1QA noted in aging cells, thus it was hypothesised that C1QA may be a Wnt activator. Targeting of the Wnt pathway has been suggested as a possible therapeutic target for the treatment of oesophageal cancer (Clément et al., 2007, Bobryshev et al., 2010). Canonical Wnt signalling is transduced through β -catenin, which is a transcriptional co-activator that transmits extracellular signals thus activating target genes such as Tcf1 and LEF1 which have been implicated in the development and progression of cancer (Roose et al., 1999,

Filali et al., 2002). This pathway is characterised by cytosolic accumulations of β -catenin which subsequently translocates into the nucleus where it binds to TCF/LEF, thus activating Wnt transcription (MacDonald et al., 2009). Evidence is mounting for targeting of the Wnt signalling pathway in the treatment of BO and OAC including the findings that nuclear accumulations of β -catenin within the oesophageal carcinogenic sequence are common, although the specific point with the highest level is currently under dispute (Moyes et al., 2012, Osterheld et al., 2002, Bian et al., 2000, Clement et al., 2006a). Results obtained in this thesis suggest that Wnt/ β -catenin signalling is significantly increased upon inhibition of C1QA expression, resulting in a significant reduction in cell viability. This finding is novel in two ways; the effect of C1QA has not been published in relation to oesophageal cancer cell proliferation nor has it been studied in relation to its role in the Wnt/ β -catenin signalling pathway.

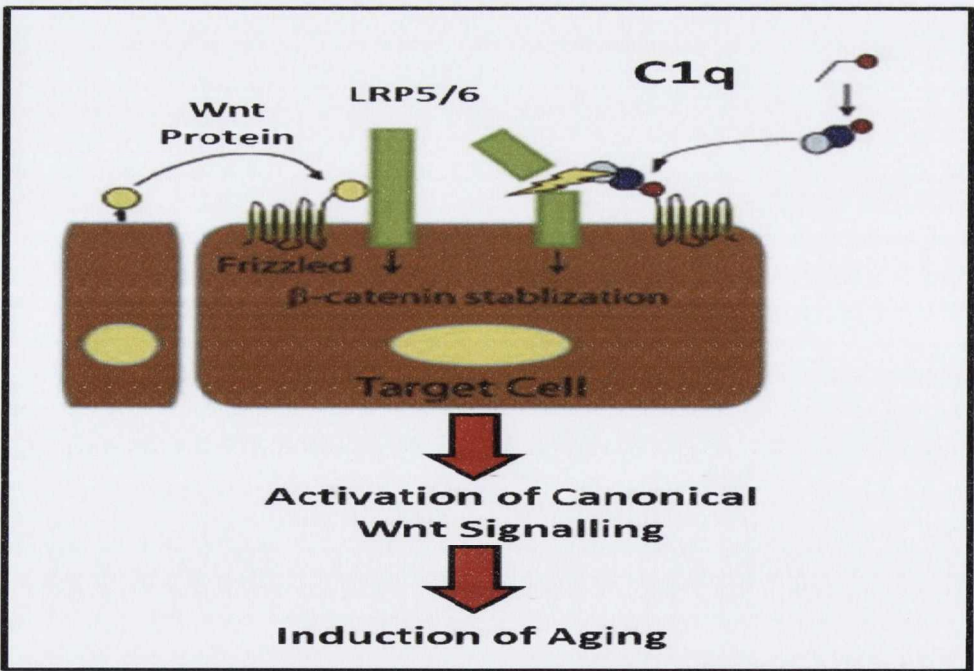


Figure 6.1: C1q Activation of Canonical Wnt Signalling Induces Aging. C1q binds to Frizzled receptors thus activating C1r/C1s, resulting in LRP5/6 cleavage and activation of canonical Wnt signalling resulting in induction of aging. (Naito et al., 2013)

Experiments undertaken to detect possible synergy between LIF and C1QA, and a panel of 56 immune related genes also highlighted RUNX2, a gene with no currently known association with oesophageal cancer, to be of particular interest in relation to oesophageal cancer. Two other genes, TREM2 and PTCRA were also identified, and interestingly all three of these genes are located within the same chromosomal region 6p21, within the MHC region. Although further validation of RUNX2 was not undertaken within this body of work, it is a gene of interest for further study as it had been highlighted in both the original screen, with a reduction in cell viability of 75%, as well as showing an association with both of our hits LIF and C1QA. Further studies by another member of the group have identified important functional effects of RUNX2 in oesophageal cancer cells.

6.1 Therapeutic Potential

Specific gene targeting has been highlighted in this body of work as a powerful *in vitro* tool to characterise the effect of individual gene silencing with respect to cell proliferation. Diagnosis of OAC is frequently late within tumour development; therefore the most common treatment would be chemotherapy and surgery, the combination of which can result in a 5-year survival of 47% as compared to surgery alone which has shown a 5-year survival of 34% (van Hagen et al., 2012, Pottgen and Stuschke, 2012). Monoclonal antibodies directed at specific molecules such as EGFR and HER2, have been used in clinical trials for treatment of gastrointestinal malignancies such as oesophageal cancer and colorectal cancer, including Cetuximab and Trastuzumab respectively (Toomey et al., 2013, Gold et al., 2010, Safran et al., 2007). Although Cetuximab had a 6-month survival of 36% in OAC this was not significant, and thus was deemed not a good candidate for second-line treatment when

administered alone (Gold et al., 2010). In a clinical trial to analyse effect of trastuzumab in patients with OAC, it was found that the median survival was 24 months, and 50% survived for 2-3 years, although this study is limited by patient numbers n=19 (Safran et al., 2007). Identification of genes which impact on cell survival could identify targets for future therapy either with small molecules or monoclonal antibodies.

6.1.1 Drug Delivery Options

Delivery of treatments to the tumour is a major issue, especially where this involves internal organs, therefore highlighting the need for new modes of specific drug delivery. Targeting a tumour with a systemic drug may be associated with significant adverse effects, but the oesophagus may prove to have more potential for targeted treatment, as it can be accessed relatively easily using an endoscope. Clinical applications of RNAi treatment have been limited by their rapid degradation, poor cellular uptake, and rapid renal clearance following systemic administration. To overcome these issues a number of methods have been developed including the use of liposomal and other nanoparticles which show great promise by improving both availability and stability of the siRNA, counteracting the negative charge of the naked siRNA, and they have also shown success *in vivo* (Ozpolat et al., 2014).

Mice have long been used as animal models, and evaluation of siRNA treatment for OSCC *in vivo* has been successful utilising xenograft technology (Sun et al., 2012). For example, mice have been subcutaneously injected with oesophageal squamous cell carcinoma cells for a week before treatment with varying siRNA including mTOR, HIF-1 α and VEGF, with or without rapamycin treatment. They were then

treated daily for 2 weeks with intraperitoneal injection of the siRNA and or rapamycin, with results showing that mTOR siRNA, with and without rapamycin treatment, significantly reduced tumour size (Sun et al., 2012). Another study pre-treated OAC cells with short hairpin (shRNA) against β 2SP and/or SOX9, which were then injected into mice and after an incubation period, tumours were either treated with or without a γ -secretase inhibitor. Tumour size was measured and results indicated that loss of β 2SP can switch the normally tumour suppressing TGF- β signalling to tumour promoting by activating Notch signalling and SOX9 (Song et al., 2013). The success of implementing these types of xenograft studies provides a wealth of information which can pave the way for clinical trials progressing to anti-cancer treatment within humans.

These methods could also be utilised for targeting of both LIF and C1QA, in murine models. A murine model of pancreatic cancer has shown that inhibition of both STAT3 and EGFR can increase apoptosis in tumour cells (Jaganathan et al., 2010). In head and neck cancer, shRNA mediated STAT3 silencing combined with cetuximab treatment (an EGFR inhibitor) can greatly decrease in cell proliferation (Bonner et al., 2011). Thus, LIF inhibition combined with an EGFR inhibitor such as cetuximab could be investigated in oesophageal cancer murine models. As previously outlined, there are a number of complement inhibitors available, but C1INH specifically targets C1. Results highlighted within this thesis indicated that combined treatment of oesophageal cells with both siC1QA and C1INH could significantly decrease C1QA protein observed via immunofluorescent staining. Thus treatment of this combined therapy in mouse models of BO and OAC may result in a reduction of tumour size to that which is resectable.

6.1.2 Translational Benefit of Targeting LIF Signalling Through STAT3 Pathway

Most tumour types express active forms of STAT3, therefore leading to its consideration as a major target for cancer therapy (Yu and Jove, 2004). Most treatments targeting the STAT pathway are aimed at the phosphorylation of STAT3 at tyrosine residue 705 (Y705), thus preventing activation of target genes. There are three main issues with targeting STAT3. Firstly, tumours over-expressing pSTAT3 are generally more resistant to genotoxic treatments and therefore may require a greater concentration or quantity of the targeting drug (Sellier et al., 2013). Another issue is that redundancy can occur among the cytokines that activate STAT3, indicating that direct inhibition of STAT3 activation would be necessary; our findings demonstrate a lack of redundancy between LIF and IL-6 suggesting that LIF may be a useful target to modulate the STAT3 pathway. Finally, continuous ablation of STAT3 in mice can cause Th1 autoimmunity; thus, targeting LIF could potentially directly inhibit STAT3 activation reducing the risk of developing autoimmunity although this remains to be tested.

Clinical inhibitors targeting EGFR, Bcr-Abl and mTOR, which are already in use, have been shown to partially inactivate the STAT3 pathway. To date, no STAT3 or LIF inhibitor is in clinical use, although several STAT3 inhibitors are known to be in varying stages of clinical trials. Currently, one commercial company in the USA is testing pSTAT3 inhibitors in three main areas; inhibiting phosphorylation of STAT3, inhibiting dimerization of pSTAT3 and inhibiting binding of pSTAT3 dimers to DNA (GLGpharma, 2014). To date, no commercially available inhibitor has been applied to the BO/OAC setting. The development of a monoclonal antibody, which binds to the

LIF receptor at the cell surface thus inhibiting LIF signalling and STAT3 activation, may be of particular interest. Inhibition of the dimerisation of the LIF receptors, gp130/LIFR, could be used to disrupt binding of LIF, thus inhibiting LIF activation. For example a truncated form of murine LIFR can also inhibit human LIF and does not alter the activity of either IL-6 or OSM (Metz et al., 2008). Therefore, this inhibitor may of particular interest to further analyse both LIF and IL-6 function in relation observation of non-redundancy between these related cytokines.

In terms of the effect that targeting LIF/JAK/STAT3 would have on patient survival, previous research in several cancer types have shown positive results, indicating that the same could be observed in and oesophageal cancer setting. The use of the JAK inhibitor, AZD1480 has been used to analyse the effect of inhibiting STAT3 activation on tumour survival and growth (Gritsina et al., 2015). For example, a study in ovarian carcinoma cells identified that treatment with AZD1480 could inhibit STAT3 phosphorylation and DNA binding, migration and adhesion of cultured ovarian carcinoma cells, while also reducing tumour growth rate, volume, and ascites production in a murine model. Additionally, AZD1480 treatment appeared to alter gene expression, decrease tumour-associated MMP activity, and reduced the suppressor T cells in the peritoneal TME of tumour-bearing mice than control mice. The authors thus concluded that inhibition of the JAK2/STAT3 pathway could lead to disruption of functions necessary for ovarian tumour growth and progression thus representing a promising therapeutic strategy (Gritsina et al., 2015). Another study aimed to evaluate the effect of the same inhibitor, AZD1480 on head and neck squamous cell carcinoma (HNSCC) preclinical models to determine its potential as a therapeutic (Chen et al., 2013c). It was identified that AZD1480 treatment could decrease cell proliferation in the majority of the HNSCC cell lines used, which

correlated to a reduction of pSTAT3 (Y705) expression. *In vivo* analysis in patient-derived xenograft models from two independent tumours identified that oral administration of AZD1480 reduced tumour growth, in both models, which also correlated with an observed decrease in pSTAT3 (Y705) expression. The authors thus suggest that JAK1/2 inhibitors could reduce STAT3 signalling and may be beneficial as a HNSCC treatment approach (Sen et al., 2015). In an oesophageal setting, using OSCC tissue samples, it was shown that positive IL-6 staining and, poor or no treatment response (IL-6 negative of 16% vs IL-6 positive of 41%; $p < 0.0001$) significantly correlated with a shorter patient survival (IL-6 negative of 3 vs IL-6 positive of 11; $p = 0.052$) (Chen et al., 2013c). Additionally, the increased IL-6 immunoreactivity noted was associated with the development of distal metastasis ($p = 0.0003$) and also a reduced treatment response rate ($p = 0.0001$). Upon inhibition of IL-6 expression tumour behaviour became less aggressive and radiation resistance was overcome both *in vitro* and *in vivo*, by increasing cell death, decreasing epithelial-mesenchymal transition, and reducing both STAT3 activation and angiogenesis in murine tumour models (Chen et al., 2013c). These studies thus suggest that inhibition of LIF/JAK/STAT3 to reduce cell proliferation in oesophageal cancer, as identified in this thesis, could work in the same manner to increase patient survival and provide a better quality of life.

6.1.2.1 Concerns Related to RNAi Targeting of LIF Signalling via the STAT3

Pathway

There are currently several clinical trials using RNAi technology, underway for the treatment of several diseases including advanced solid cancers. One of the main issues with using siRNA to target tumours is the need for its continual administration to the

target as they have very short lives; to overcome this shRNAs can be used. Given this, data regarding RNAi-targeted silencing of STAT genes for cancer therapy are currently limited to *in vitro* studies and *in vivo* studies of animal models only (Kunigal et al., 2009, Ma et al., 2011, Kaymaz et al., 2013). Several studies (reviewed by Yu and Jove, 2004), have highlighted the fact that inhibiting or decreasing the levels of STAT3 can induce apoptosis in cancer cells, while healthy cells were able to continue growing. Given this, the work presented in this thesis may be of benefit following analysis with clinical samples, and subsequent animal models, either using frequent administration of siRNA or using a shRNA targeting LIF.

6.1.3 Translational Benefit of Targeting Complement and the Wnt Pathway

Rituximab is a CD20 targeting monoclonal antibody (mAb), which is widely used in the treatment of non-Hodgkin's lymphoma where it lyses lymphoma cells. It induces complement dependent cytotoxicity (CDC), thereby activating the complement induced immune response, and is also dependent on C1q as shown in *C1q^{-/-}* mice (Di Gaetano et al., 2003). Our data suggest that there may be other mechanisms whereby complement could influence tumour proliferation other than through conventional complement-mediated cell lysis pathways. Although Rituximab activates complement, following our findings, we would need to utilise a complement inhibitor in order to reduce oesophageal cancer cell viability. There are a number of complement inhibitors available that target the three complement pathways at different points, although only one has been used to inhibit C1. C1INH is a serine protease inhibitor that binds to and inactivates C1r and C1s thereby inhibiting complement initiation of both the classical and lectin pathways.

Several other recent studies have shown that complement activation may promote carcinogenesis, for example C5a can suppress the anti-tumour T-cell response by recruiting Treg and MDSC (Corrales et al., 2012, Markiewski et al., 2008), while inhibition of complement can reduce tumour growth by altering endothelial cell function and VEGF expression in ovarian cancer (Nunez-Cruz et al., 2012). A more recent study has also highlighted that inhibition of complement, in conjunction with radiation therapy may further reduce tumour size as compared to either alone. This study hypothesized that in the context of radiation therapy, inhibition of complement activation may act to improve the therapeutic response by interfering with the phagocytic uptake of apoptotic cells, which would lead to a higher level of necrotic burden and also the formation of a more immunogenic tumour environment (Elvington et al., 2014). The complement inhibitor, CR2-crry, which acts by inhibiting all complement pathways at the C3 activation step, was utilised in the treatment of a murine lymphoma model. In this context co-treatment with both radiation therapy and CR2-crry significantly decreased tumour size ($p < 0.001$) and improved therapeutic response ($p < 0.001$) by increasing tumour cell apoptosis and inflammation, as compared to untreated, radiation treated only and CR2-crry treated only. The median survival of the mice receiving the combined treatment was significantly higher than the mice receiving radiation alone (28 days vs 17 days), while the mean tumour size was also significantly reduced at both day 11 and at the time they were sacrificed for mice receiving the combined treatment as compared to radiation therapy alone. Interestingly, 8 out of the 11 mice that received the combined treatment had no detectable primary tumour at time of sacrifice (Elvington et al., 2014). Combined, these studies support the findings of this thesis, that inhibition of a

complement component may in fact have an anti-tumour effect, to help decrease tumour size and increase patient survival.

The recent association noted between C1q and the Wnt pathway may pose an interesting target for treatment of OAC through complement, although this is a relatively new concept, where addition of C1q increases Wnt/ β -catenin signalling in ageing cells (Naito et al., 2012). This finding contradicts what has been shown in this thesis, where C1QA silencing results in a reduction in OAC cell proliferation while increasing β -catenin stabilisation, providing a different possible model to that described for its action in ageing studies. Modulation of β -catenin is a promising target for anticancer therapy, as demonstrated by its inhibition using siRNA resulting in a decrease in colon cancer cell viability (Verma et al., 2003). As most of the research linking Wnt/ β -catenin signalling to cancer is in relation to inhibiting β -catenin stabilisation, a new area of research would be to investigate the role of β -catenin overexpression within BO through the classical complement pathway. In this regard, in a clinical setting, it has been shown that the canonical Wnt pathway is activated early during the progression to OAC, with β -catenin stabilisation occurring in the progression from BO to OAC. IHC staining indicated that high-grade dysplastic cells show the least amount of nuclear β -catenin as compared to low-grade dysplastic (LGD) or OAC samples (Bian et al., 2000), with the highest accumulation noted in the transition from metaplasia to LGD. Although this finding was contradicted in 2012 when it was shown that the highest levels of nuclear β -catenin were shown in HGD patient samples (Moyes et al., 2012). Interestingly, in mouse fibroblasts the nuclear accumulation of β -catenin was indicative of significantly better survival (61% vs 26%, 1-year survival) (Osterheld et al., 2002), while over-expression of β -catenin

can also induce apoptosis over time (Kim et al., 2000). This suggests that the effects of β -catenin are complex and potentially bi-directional in OAC cells; hence, treating a patient with a drug inhibiting C1QA which results in stabilisation of β -catenin at this point, could paradoxically be a novel yet effective treatment of BO. Utilisation of small interfering RNAs (siRNA) to inhibit Wnt1, Wnt2, Dvl and β -catenin can also induce apoptosis, decrease β -catenin levels and reduce tumour size in a variety of cancer types including mesothelioma, NSCLC and colon cancer respectively (You et al., 2004, Mazieres et al., 2005, Uematsu et al., 2003, Verma et al., 2003). Therefore there are potential benefits of targeting the Wnt pathway components in the treatment of cancer, and the use of gene inhibition using siRNAs has been successfully demonstrated within cancer research.

Analysis of nuclear β -catenin in cell lines spanning the oesophageal carcinogenic sequence, with and without C1QA siRNA treatment, would be a simple step to determine if the accumulations seen in the HGD cells line, GOhTRT, are associated with tumour stage, thus determining the point at which C1QA may start to have an effect on β -catenin levels. Upon activation, Wnt proteins bind to the Frizzled-LRP5/6 receptor complex resulting in recruitment of Axin to LRP5/6, leading to β -catenin stabilisation. In the study by Naito et al., it was shown that C1q binds to Frizzled receptors, thus activating the canonical Wnt pathway by inducing C1s-dependent cleavage of LRP6, leading to β -catenin stabilisation (Naito et al., 2012). As our study indicated that inhibition of C1QA also resulted in β -catenin stabilisation, it is an obvious step to analyse the effect on LRP5/6 cleavage within our oesophageal cells. This could be undertaken using different methods including western blot, ELISA and

luciferase assay to detect both cleavage and levels of LRP5/6 both before and after C1QA siRNA treatment.

6.2 Future Directions

The successful completion of the siRNA library screen resulted in identification of both LIF and C1QA as siRNA targets with the potential to significantly inhibit OAC cell proliferation. The identification of the role that they play in the STAT3 and Wnt pathways compels further investigation as targeting these pathways may be a novel application in the treatment of OAC.

LIF signalling can activate STAT3, which can up-regulate the pro-survival Bcl-2 family genes, for example STAT3 is known to up-regulate Mcl-1, thereby decreasing apoptosis (Stephanou et al., 2000, Zhuang et al., 2007). Therefore, investigation of possible synergy between Bcl-2 family genes and LIF, thus further decreasing the level of cell viability in cell lines spanning the oesophageal carcinogenic sequence, may be of particular interest. VEGF is a cytokine that can stimulate angiogenesis thus, providing a blood supply to the tumour and potentially increasing its growth, while Src activation is known to be significantly increased in BO, and further increased in OAC (Kumble et al., 1997). Nuclear localisation of STAT3 is associated with increased VEGF expression in pancreatic cancer (Wei et al., 2003), and tumours displaying aberrant Src activation commonly over produce VEGF, which can be blocked by STAT3 inhibition (Laird et al., 2003). Thus, examination of LIF inhibition on angiogenesis via VEGF regulation may be of interest in the oesophageal carcinogenic sequence.

Gene silencing by promoter methylation can result in β -catenin stabilisation and nuclear accumulations, thus analysis of the level of methylation of these genes, before and after C1QA inhibition by siRNA, may help to elucidate further the mechanisms behind the apparent increase in β -catenin noted throughout this thesis. A number of genes within the canonical Wnt pathway are known to be hypermethylated in BO and OAC, resulting in gene silencing including APC, SFRP1 and WIF1. Adenomatous polyposis coli (APC) is a negative regulator of the canonical Wnt pathway, and loss of APC is known to increase tumourigenesis by decreasing apoptosis and increasing cell survival. APC promoter methylation has been shown in 68-95% of OAC patients and is known to occur early within the oesophageal carcinogenic sequence, during metaplasia (Sarbia et al., 2004, Clement et al., 2006b). This was also demonstrated by the use of a siRNA targeting APC resulting in nuclear accumulation of β -catenin in an OAC cell line (Clement et al., 2006a). Thus it may be of interest to detect the level of APC within our cell lines, before and after treatment with C1QA siRNA. SFRP1 also shows promoter methylation in nearly 90% of OAC patients (Clement et al., 2006a), and the same has been noted for WIF1 but with higher levels noted in BO than in OAC (Clement et al., 2008). Following the above analysis, detection of methylation levels within each of the genes mentioned, APC, SFRP1 and WIF1, could be undertaken to determine if epigenetics may also be involved in increased β -catenin following C1QA inhibition. To further determine if β -catenin stabilisation is specific to C1QA inhibition, silencing of the other components of C1q; C1QB and C1QC, would also be an important step, and could be performed using the techniques outlined in chapter 5 of this thesis, including MTT assay, RT-PCR, Western blot and luciferase assay.

There is mounting evidence of cross-talk between the Wnt/ β -catenin pathway and the STAT3 pathway. Research has shown that in OSCC, 95.7% of patients with nuclear β -catenin also showed STAT3 overexpression ($p < 0.001$), and that of these, 81% showed nuclear STAT3 staining, with subsequent analysis indicating that β -catenin overexpression upregulates STAT3 mRNA and protein (Yan et al., 2008). Another interesting finding indicated that in mouse embryonic stem cells (ESC), Wnt/ β -catenin activation can increase STAT3 levels, and that LIF and the Wnt proteins, including Wnt3a, have a synergistic effect as both can regulate STAT3 within these cells by phosphorylation of STAT3 and increasing Stat3 mRNA respectively (Hao et al., 2006). Results highlighted following the synergy experiment outlined within this thesis indicated that cell viability was decreased upon double knockout of LIF and C1QA by 34% ($p < 0.05$). This suggests that although cell viability was not significantly decreased indicating a synergistic interaction, the relationship between Wnt/ β -catenin and STAT3 may still be interesting to analyse in the oesophageal cancer setting, by both addition and inhibition of LIF, via Western blot. In a similar fashion, the interaction between C1QA induced β -catenin stabilisation, and LIF induced STAT3 activation may be worthwhile to examine although the results may differ from those in other cell types.

Following detection of synergy between LIF and C1QA, and a panel of immune-related genes, three genes were highlighted, PTCRA, TREM2 and RUNX2. PTCRA is a direct target gene of Notch1 (Liu et al., 2010), and in oesophageal cancer, inhibition of Notch-1 expression can reduce both epithelial-to-mesenchymal transition (EMT) and, invasion and metastasis, in OSCC cells (Wang et al., 2014). Thus investigation of alterations within the Notch pathway and downstream effects

following PTCRA inhibition may also be of interest in OAC. Genetic variants in the PI3K/PTEN/AKT/mTOR pathway can mediate oesophageal cancer patient survival (Hildebrandt et al., 2009), while recent evidence demonstrated that ligation of TREM2 can activate PI3K and prevent apoptosis in bone marrow macrophages (Peng et al., 2010). This indicates that it may be of interest to investigate TREM2 inhibition on the PI3K pathway within oesophageal carcinogenesis. Additionally, a recent study has demonstrated that in oesophageal cells, inhibition of the PI3K pathway with wortmannin, can mediate the IL-4 driven decrease in both K8 and K13 which are indicative of BO and normal oesophagus respectively (Shan, 2014).

There is no published data regarding RUNX2 and either BO or OAC, therefore the role that RUNX2 plays in the oesophagus would need to be determined. One possible route of investigation could be in relation to its role in angiogenesis and advanced tumours, such as VEGF, a known angiogenic factor, that can be induced by both over-expression of RUNX2 and HIF1 α individually, as well as in combination (Kwon et al., 2011). Both VEGF and HIF1 α are expressed at high levels in BO, OSCC and OAC, especially in the more advanced tumours of stage T3 and T4, although HIF1 α is more highly expressed in OSCC (Goscinski et al., 2013). However, an increase in HIF1 α is noted in BO as compared to normal squamous epithelium, although this did not increase during progression to OAC, suggesting that HIF1 α is associated with the inflammatory response associated with BO development (Ling et al., 2009). In relation to the association of LIF and angiogenesis, reports are conflicting about its effect on VEGF; one study reports that LIF can reduce VEGF expression in astrocytes while another reports that activation of gp130 by LIF increases VEGF mRNA levels in cardiomyocytes resulting in STAT3 activation (Kubota et al., 2008, Funamoto et

al., 2000). There are currently no publications indicating the effect of LIF on VEGF and angiogenesis in the oesophageal setting, although it has been shown that silencing of IL-6 can reduce the levels of VEGF as shown in a murine model of OSCC (Chen et al., 2013c). Thus, it may be postulated that LIF inhibition may also reduce angiogenesis, which may be further induced by silencing of both LIF and RUNX2. From the synergy experiment performed in this thesis, it was shown that the combination of LIF and RUNX2 reduced cell viability by 55% ($p=0.006$), indicating that there may be an interesting association between these two genes worth pursuing. This may also be important in the investigation into the lack of redundancy between LIF and IL-6. It has long been thought that these worked in parallel rather than in series as this is observed between other members of the IL-6 family of cytokines, but from the research presented within this project, this does not appear to be the case for LIF and IL-6. As it has been reported that IL-6 inhibition reduces VEGF level, it may also be of interest to investigate the outcome of LIF inhibition in relation to both the effect on VEGF and on IL-6 levels within oesophageal cells and clinical tissue samples. To further analyse the possible synergistic effect between C1QA and RUNX2, it may be of interest to investigate the WW domain-containing oxidoreductase (WWOX/WOX1). This is a tumour suppressor which is attenuated in some human cancers. Furthermore, WWOX can reduce RUNX2 expression in osteosarcoma cells (Kurek et al., 2010) while treatment of prostate cancer cells with C1q can induce apoptosis through activation of WOX1, which destabilises the adherence of the cells (Hong et al., 2009). Thus the effect of both, inhibition of C1QA and addition of C1q from human serum, on OAC cells could be investigated.

One of the main issues with this study is that it was performed in cell lines, rather than in tissue samples, and that clinical samples were not utilised for further validation of the findings. Patient samples are a better representation of cancer rather than cell lines, as in tissue samples you have a tumour microenvironment, as previously discussed. Cell-to-cell signalling and inflammatory infiltrate play key roles in the formation and progression of a tumour, which are not generally represented in cell line use. Thus the use of patient biopsy samples are invaluable to scientific research and obtaining these tissue samples could greatly help in the further elucidation of the findings in this thesis. They would give a more realistic representation of gene expression, growth factors and proliferation markers. For example, using RT-PCR to determine the expression levels of both LIF and C1QA in samples spanning the oesophageal carcinogenic sequence would add clinical context to the cellular findings observed. The data generated would be of particular importance as it would identify if either gene is upregulated in HGD and/or OAC as compared to normal or BO, both of which has been suggested by the data generated in the Oncomine database as outlined previously (Section 4.3.7). The use of animal models is also a logical next step in validating the findings of this thesis. For example, mice treated with CTLA4^{APT}, a siRNA covalently linked to an aptamer to inhibit STAT3, were sacrificed, and when sections of the tumours were stained with Ki-67, a commonly used cell proliferation marker, it was found that there was a significant decrease in cell proliferation ($p < 0.001$) as compared to the untreated tissue (Herrmann et al., 2014). Staining for Ki-67 following inhibition of LIF or C1QA in murine models could be compared to untreated tumour sections to analyse any difference in cell proliferation, and while also staining to analyse the gene expression levels of both genes.

6.3 Concluding Remarks

In all, we can conclude that the screen itself has been successfully optimised for purpose and implementation, while extensive statistical analysis was undertaken to reduce the risk of off-target effects and false positive rates, showing positive results. Following this, 12 genes were identified, of which three were selected for further study; LIF, C1QA and RUNX2, that appear to play a key role in oesophageal cancer cell proliferation, and silencing them could result in a reduced rate of OAC proliferation, therefore therapeutically, improving the quality of life of patients by reducing the rate of growth of a tumour. The success of using RNAi technology to target genes known to be up-regulated in cancer has shown potential in both *in vitro* and *in vivo* but as yet clinical trials are limited. With this in mind, it is positive to think that targeting one or more of the genes identified by this screen could be used with a successful outcome for OAC patients.

Chapter 7

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7 Bibliography

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Chapter 8

Appendix

8 Appendix

8.1 Plate Layout for Immune Panel Screen

	1	2	3	4	5	6	7	8	9	10	11	12
A	Resting	Resting	Resting	Resting	Resting	Resting	Resting	Resting	Resting	Resting	Resting	Resting
B	Resting	Neg Ctrl	MNDA	NFKBIL2	C1R	TRAF4	TRPV2	IL15RA	IL9R	TLR2	GPC3	PTCRA
C	Resting	Neg Ctrl	RABGEF1	RUNX2	C1S	KIF1C	BMP3	IL17	ITGB3	THBS2	HF1	STAT3
D	Resting	Neg Ctrl	TF	CES1	TGFB2	HLA-G	CD5	IL22	RELA	BCL9	IL13	Resting
E	Resting	GATA6	CSEN	GMPR2	GSTZ1	LIF	ICAM1	IL2RA	PROK1	C1QA	LIFR	Resting
F	Resting	GATA6	MAD	PIP5K1B	TP53	TNFRSF8	IFNW1	IL6	TGFB1	C1QB	IFNA7	Resting
G	Resting	GATA6	ILF3	ABP1	BMP1	TREM2	IL11	IL6ST	THBD	C1QC	NK4	Resting
H	Resting	Resting	Resting	Resting	Resting	Resting	Resting	Resting	Resting	Resting	Resting	Resting

8.2 R Code for Generation of Interpretable Output File from Raw Screen Data

```
#####  
#To Generate Dataset File #  
  
generateDatasetFile("DruggableGenomeScreen", "DGScreen in GohTRT cells",  
NA_character_, "RNAither_output.xls", platelayout1, platelayout2,  
8, 12, 1, emptyWells, poorWells, controlCoordsOutput, backgroundValOutput, meanSignalOutput,  
SDmeansignal, objNumOutput, cellnumOutput)  
header <- readLines("RNAither_output.xls", 3)  
dataset <- read.table("RNAither_output.xls", skip = 3, stringsAsFactors = FALSE)  
# To join dataset files #  
  
CombinedData<-joinDatasets(list(dataset1, dataset2, dataset3))  
  
#####  
# Normalization to negative controls #  
  
Norm<-control[Norm(header, CombinedData, list(2, 0, "SigIntensity", 1))  
NormHeader<-Norm[[1]]
```

```

NormDataset<-Norm[[2]]
#####
# Stats and Graphs on Data #

makeBoxplotControls(header, NormDataset, "SigIntensity", "Data vs. Controls", 1, 1)

plotControlHistoPerplate(header, NormDataset, "SigIntensity", "ControlHisto", 2, 1)

ZPRIMEQualControl(header, NormDataset, "SigIntensity", "Z' factors per plate", 1)

controlDensity(header, NormDataset, "SigIntensity", "Control density", 1, 1, 1)

plotQQperplate(header, NormDataset, "SigIntensity", "QQplot", 1, 1)

#####
# P-Value Calculations #
pvals <- Ttest(NormDataset, list("two.sided", 1, "SigIntensity", "GeneName"))
pValVec <- pvals[[1]]
scoredDataset <- pvals[[2]]

```



```
#####
# Normalization by Z Score #

ZS<-ZScore(header1, NormDataset, list("SigIntensity", 0))
Zheader<-ZScore[[1]]
Zdataset<-ZScore[[2]]

#####
# SSMD ranking #

NegCtrlSubset<-createSubset(RNAither_output, RNAither_output$NegCtrl, NegCtrl)
d<-(d1+d2+d3)/3
s<-sqrt(((d1-d)^2+(d2-d)^2+(d3-d)^2)/2)
ssmd<-(gamma(1)/gamma(1/2))*(sqrt(2/2))*(d/s)
scoredDataset<-ssmd
```

8.3 LIF Sequences

Key:

Exons/Introns

Flanking sequence

UTR

Intron sequence

Translated sequence

Table 8.1: Exon Sequences of LIF Transcript 1 (LIF_001)

No.	Exon / Intron	Start	End	Start Phase	End Phase	Length	Sequence
5' upstream sequence							tcactctcaactttttccatttcataatttcctatgatgcacctcaaaaa
1	ENSE00001551728	30,642,840	30,642,666	-	1	175	CTTCCTGGA CTGGGATCCGGTAAATATAGCTGTTTCTGTCTTACAAACACAGGCTCCA GTATATAATCAGGCAAAATCCCATTTGAGCATGACCTCTGAAAACTGCCGGCATCTG AGGTTTCCTCCAAGGCCCTCTGAAGTGCAGCCCATATGAAGGTTTGGCGGCAG
	Intron 1-2	30,642,665	30,640,923			1,743	gtaaatacacccgcgccgcgcggc.....atggcttcttctgaetgtccccag
2	ENSE00000879785	30,640,922	30,640,744	1	0	179	GAGTTGTGCCCTGCTGTTGGTTCTGCACTGGAACATGGGGCGGGAGCCCCCTCCCCA TCACCCCTGTCAACGCCACCTGTGCCATAGCCACCCATGTCAACAACCTCATGAACC AGATCAGGAGCCAACTGGCACAGCTCAATGGCAGTGCCAATGCCCTCTTATTTCTAT
	Intron 2-3	30,640,743	30,640,051			693	gtaagttacccctgggataactgaca.....acgtcaectcccttctgccccctcag
3	ENSE00001215846	30,640,050	30,636,436	0	-	3,615	TACACAGCCAGGGGAGCGGTTCCCCCAACAACCTGGACAAGCTATGTGGCCCCAACGTG ACGGACTTCCCGCCCTTCCAGCCAAACGGCACGGAGAAGGCCAAAGCTGGTGAGCTGTAC CGCATAGTGTGTAACCTTGGACCTCCCTGGCAACATCACCCGGGACCAGAGATCCTC AACCCAGTGCCCTCAGCCTCCACAGCAAGCTCAACGCCACCCGCGACATCCTGCGAGGC CTCCTTAGCAACGTGTGTGCCGCTGTGCAGCAAGTACCACGTGGGCCATGTGGACGTG ACCTACGGCCCTGACACCTCGGTAAGGATGCTTCCAGAAGAAGCTGGCTGTCAA CTCCTGGGAAGTATAAGCAGATCATCGCGGTGGGCCAGGCCCTCTAGCAGGAGGTC TTGAAGTGTGCTGTGAACCGAGGATCTCAGGAGTTGGTCCAGATGTGGGGCCTGTCC AAGGGTGGCTGGGGCCAGGGCATCGCTAAACCCAAATGGGGGTGCTGGCAGACCCCCGA GGGTGCCCTGGCCAGTCCACTCTGGGCTGGGCTGTGATGAAGCTGAGCAGATGGA

No.	Exon / Intron	Start	End	Start Phase	End Phase	Length	Sequence
							AAC TTC ATAGG GAGGAGCTAGAAGAGGTGCCCCCTTCCTTGAGATGTGACTGG GGAGCGTGGGCTGGACTTCTGCCCTTACTTGTCCCTTTGGCCCCCTTGCTACTTTGTGA GTGACAAACTACACAGTCACTCTACAGAGCCCTACACAGAGGTGAGCAGCAGGGCC CAGGGAGTGGACCAGCCCCAGCAATATACCACTGTGCTTGTGCTGCCCTTAGG TTGGACTTAGTGGCCAGAGGGGCTAGGATCCAAAGACTCCTGTCCCTTAGAAT TTGATGAGTGGAGATAGAGAGGGGCTCTGGATGGAAGCTGTCTCTTTGAGATG ATCAGAGAACTTGGGCATAGAACAACTGGCAGAGTTTCGAAGAGAGTCACTTGGC ATTGAGGCTCTTGGGAGGCGAGACCACTTGAGCCCTGGGAAGAGACACTGGGA GGAGGAGAGGCTGGAAGCTTTGTAGTCTCTGTTCTCTCCCGTAGTCTCCCTG CAGCCTGGATGGCCAGGCTGTATGGCTGGACCTGCAGAGGGGTTGTGAGGTGGT AGGGCAGGGGCAAGTTGCTAAGTCAAGTGCAGAGTTCTGAGGACCCAGGCTCTCCTC TGGGTAAGGCTGTGAAGAAGGGGCTGGGGTAGTCAGATGACAGCTCACTTGAGGC CTGGGAGGCTTGTAGGTCACACAGAGTACTTGAAGGGGACTGAGGGCGTCTAGG TCCCCAGGGCAGGGAACAGCAGAACTTAGGTCAGGCTCAAGGAACCTGAGCTCA AGCTGCTGTGCGTGTGACCTGGCATGATTTCTATTATGATATCTTATATTA ACTTATGTGCTTTAGTGGCCAGTTAATTCCTTTCCCTGTGCTCCTCACTCAACAA ATATGATGATGGCTCCCGACACAGCGCCAGGGCTTAGCAGGGCTGTGCTGGA AGTCACAATGTACAGTGAATAAGCCTTACGGTGAAGTCAGAGAAGGTCGGATC TGAGAGATGGGAGGCCGTGAGTGGAGTGGGGGCTTGCTCAACCCGCCATCCCC TACTGTACTTGCCTTAGGGTGTCAAGGTCAGGCTGCAGGGCTGGGCCAATTTGTGA GAGCGGGTGCCTTCTGTGTGATTCAGGGGCTGTTCACACTGTCTTGGGGCC CCAGCATTTGTGTGAGGGCAGCTTCTTGACATATTTGCCCCCTGGAGCAGTGG GCAAGACAGTCTTGTGGCCACCCGTCTCTGTCTGTGTGCCCATGTGCTCTGA ATAGCGCCCTGGAACAACCCCTGCCCTGCACCAAGCATGCTCCGACAGCAGGAAGCT CCTCTGTGGCCGGACACCCATAGACGTTGGGGGGGCTGGCTGGGCCAGACCCAGG AAGTGGGGTAGACTGGGGGATCAGCTGCCCATTGTCTCCAGAGAGGAGGAGG TGCAGATGCTGGACTCAGACAGGAAGCTGTGGCCCTCTGCTCCACCCCATCCA CTCCACCCATGTGTGGCTCCAGGAGGGAACCGATCTTCTCTTGTGTGGGGCC AGCGAGTGGAGAAACGCCCTCCAGTCTGAGAGCAGGGGAGGGAAGGAGCAGACTT GGGCGAGCTGCTGAGAGCAGTGTTCGCTTCTTCAAAACCTGAGCGGCTGCCGCC TCCAAGTTCCTCCGACAAGATGATGTACTAATTATGTACTTTTCACTTCTTGACCT TTTCCCTGTGCTCTCTAAGCACTTACTTGATGGCGGTGGGCAAGTGTCCAGGCAAGT CCTGAGGCTTGGGTTGGGGTGGAGGGTGGCCGGAGTTGCATGTGCATGCCAA CAGCAAGCAGAGGATGTGGCTGTGAGATGTGGCCACACTACCTTGTCCAGGATGA GGACTGCTTCTCCTCTCTGCTTCACTCCGCTTAGCTTGGGCTGGCTGCAATCCCCA GGATGGCTTGAGAAAGACAACTGTCTGAAACAGAGTTGCTGATTCACCCGGGG GGCCGGCTGACTGCCCATCACTCACTCTCCCTGTGGAAGTGTGTGCCAGGC

No. Exon / Intron	Start	End	Start Phase	End Phase	Length	Sequence
						CCACCTTGGGGCCCTGGCTCTGAGTCGCTCTCCCACCCAGCCTGGACTTGGCCCCCATGGG ACCCATCCTCAGTGTCCCTCCAGATCCCGTCCGGCAGCTTGGCGTCCACCTGCACAGC ATCACTGAATCACAGAGCCTTTGCGTGAAACAGCTCTGCCAGCCGGGAGCTGGGTTTCT CTTCCCTTTTATCTGCTGGTGGACCACACCTGGCCTGGCCGAGGAGAGAGAGTT TACCAAGAGAGATGTCCTCGGGCCCTTATTATTATTAAACATTTTAAAAAGCACT GCTAGTTTACTTGTCTCTCTCCCCCATCGTCCCCATCGTCTCTGTCCCTGACTTGGG GCACCTCCACCCCTGACCCAGCCAGTCCAGCTCGCTTGGCGCTCTCCAGATAGACAT AGTGTGGGGTTGGAGCTCTGGCACCCGGGGAGGTAGCATTTCCCTGCAGATGGTACAG ATGTTCCCTGCCCTTAGAGTCACTCTAGTTCGCCACCTCAATCCCGCATCCAGCCTTCAG TCCCGCCACGTGTAGTCCGTGGGCCACCGTGGGCCCTTAGAGGTTTCCTCCTTCC TTTCCACTGAAAAGCACATGGCCTTGGTGACAAATCCTCTTTGATGAATACCTGT GGGATGTTTCATACTGACAGATTATTTTATTATTCAATGTCATATTTAAAAATATTTA TTTTTTATACCAAAATGAATACTTTTTTTTTTAAGAAAAAAGAGAAATGAATAAGAAT CTACTCTGGCTGGC
3' downstream sequence						tctccggagtgtaactgatgtggggagatgggctggaagggtgggactgt.....

Table 8.2: Exon Sequence of LIF Transcript 2 (LIF_002)

[illegible]

Each of the 4 sequences supplied by Dharmacon that are contained within the pooled siRNA are highlighted within the Ensembl sequences in table 7.1 and 7.2.

8.4 C1QA Sequences

Key:

Exons/Introns

Flanking sequence

UTR

Intron sequence

Translated sequence

Table 8.3: Exon Sequence of C1QA Transcript 1 (C1QA_001)

No.	Exon / Intron	Start	End	Start Phase	End Phase	Length	Sequence
5' upstream sequence							tgcccccaactccacacaccagctgttgctggggcagcgcaccaatgtccc
1	ENSE00001413929	22,962,999	22,963,195	-	-	197	AGTCTTGCTGAAGTCTGCTTGAAATGTCCCTGGTGAGTTCTTGGCCACTGGGGAAGTTCA GGGGCAGGTCTGAAGAAGGGGAAGTAGGAAGGGATGTGAACCTTGGCCACAGCCTGGAG CCACTCCTGCTGGGAGCCCCACAGGGTCCCTGGGGGAGGCGAGAGCATCCAGTTGGAG TTGACACAGGAGGCAG
Intron 1-2							gtgaggccagagteccagagggagg.....cccaggccctcccgtgtctccacag
2	ENSE00002696914	22,964,103	22,964,272	-	1	170	AGGCATCATGAGAGGTCCCGGGGATGGTGGTGCTCTGTGTGCTGGCCATATCGCTGGC CTCTATGGTGACCGAGGACTTGTCCGAGCACCAGACGGGAAGAAAGGGAGGCAGGAAG ACCTGGCAGACGGGGCGGCCAGGCCTCAAGGGGGAGCAAGGGGAGCCGG
Intron 2-3							gtaagcaaccttcctcgggaaccag.....tccaatctggcatttctccccacag
3	ENSE00001548747	22,965,326	22,966,101	1	-	776	GGGCCCCTGGCATCCGACAGGCATCCAAGSCCTTAAGGAGACCAGGGGGAACCTGGGC CCTCTGAAACCCCGCAAGTGGGTACCCAGGCCAGCGGCCCTCGGAGCCCGTG GCATCCCGGAATTAAAGGCACCAAGGGCAGCCCAGGAACATCAAGACCAGCCGAGGC CAGCCTTCTCCGCCATTCCGGGAACCCCAATGGGGCAACGTGGTCATCTTCGACA CGGTATCACCAACCAGGAAGACCGTACCAGAACCACTCCGGCCGATTCTGTCACCTG TACCCGGCTACTACTACTTCACTTCCAGTGTGTCTCCAGTGGGAAATCTGCCTGTCCA TCGTCTCCTCTCAAGGGCCAGTCCGACGCTCCCTGGCTTCTGTACACCAACCAACA AGGGGCTCTCCAGGTGGTGTCAAGGGGCATGGTTCAGTGTGAGAGGGTGACACAGG TCTGGGTTGAAAAAGACCCCAAAAGGGTCACATTTACCAAGGGCTGTAGGCCGACAGCG

No. Exon / Intron	Start	End	Start Phase	End Phase	Length	Sequence
3' downstream sequence						TCTTCAAGCGGCTTCCTCATCTTCCCATCTGCTGAGCCAGGGAGGAGACCCCTCCCCAC CCACCTCTCTGGCTTCATGCTCCGCTGTAAATGGGGCGCTATTGCTTCAAGTGTG AAGGAGGGGGCTGGCTCTGAGAGCCCGCAGGACTGGCTGCCCCGTGACACATGCTCTAAG AAGCTGCTTTCTTAGACCTCTTCCTGGAATAACATCTGTGTCTGTCTGCTGAA catgagcttcaagttgctactcgagcattgagaagygacctaaataa.....

Table 8.4: Exon Sequence of CIQA Transcript 2 (CIQA_002)

No. Exon / Intron	Start	End	Start Phase	End Phase	Length	Sequence
5' upstream sequence						gaagccccagaagygagggctgcagagttctgggaaccagggagctggc CCAGAGGCTGGCAGCTGAGGCGAGGAGGGAGGAATACTGTGCTGATTCTAGGAC AGGATGGGGTGCACACTGATCCAGGACCCAGAAATGG gtgaagtggaggaaccttaactcctt.....cccaagccctcccgtygtctccacag
1	ENSE00001666127	22,963,255	22,963,353	-	99	CCAGAGGCTGGCAGCTGAGGCGAGGAGGGAGGAATACTGTGCTGATTCTAGGAC AGGATGGGGTGCACACTGATCCAGGACCCAGAAATGG
Intron 1-2	22,963,354	22,964,102			749	gtgaagtggaggaaccttaactcctt.....cccaagccctcccgtygtctccacag
2	ENSE00002696914	22,964,103	22,964,272	-	170	AGGCATATGAGAGGCTCCCGGGGATGGCTGTGCTGTGTGTGCTGGCCATATCGCTGGC CTCTATGTGACCGAGGACTTGTGCGGACACGAGCGGAAAGGGAGGCAGAGAAAG ACCTGGCAGACGGGGGCGGCCAGGCTCAAGGGGAGCAAGGGGAGCCCG
Intron 2-3	22,964,273	22,965,325			1,053	gtaagcaccccttcctcgggaccacag.....tccaatctgycatttctccccacag
3	ENSE00001636247	22,965,326	22,965,824	1	499	GGGCCCCTGGCATCGGACAGGCATCCAGGCCCTTAAGGAGACCAAGGGGAACCTGGGC CCTCTGGAAAACCCGGGCAAGGTGGGCTACCCAGGGCCAGCGGCCCTCGAGGCCGCTG GCATCCGGGAATTAAGGACCAAGGGCAGCCAGGAAACATCAAGACCAAGCCGAGGC CAGCTTCTCCGCCATTCCGGGGAACCCCAATGGGGGCAACGTGTGTCATTGGACA CGGTCAATCACCAACCAGGAAGAACCGTAACAGAAACCACTCCGGCCGATTGCTCTGCACTG TACCCGGCTACTACTACTTCACTTCCAGGTGCTGCCAGTGGGAAATCGCCTGTGCA TCGTCTCTCTCTCAAGGGGCCAGGTCCGACGCTCCCTGGGCTTCTGTGACACCAACCAACA AGGGGCTCTTCCAGGTGCTGTCAGGGGCAATGCTTCAAGCTGCACGAGGGGTGACCAAG TCTGGTTGAAAAAGACCC
3' downstream						caaaaagygltcacatttaccagggctctgagggccgacagcgtcttcaagcg.....

No.	Exon / Intron	Start	End	Start Phase	End Phase	Length	Sequence
	sequence						

Table 8.5: Exon Sequence of C1QA Transcript 3 (C1QA_003)

No.	Exon / Intron	Start	End	Start Phase	End Phase	Length	Sequence
	5' upstream sequence						
1	<u>ENSE00001553648</u>	22,964,073	22,964,272	-	1	200	ttgtgtgcatgggactcaagggtggagctgggtgtgagtgtgatgtcca ACTTGCCAGGCCCTCCCGTGTCTCCACAGAGGCATCATGGAGGTCCCCGGGGATGGCT GGTGCTGTGTGTGGCCAATCGCTGGCCTCTAAGGTACCCAGGACTTGTGCCGAGC ACCAGACGGGAAGAAAGGGAGGCAGGAAGACCTGGCAGACGGGGCGGCAGGCCTCAA GGGGAGCAAGGGAGCCCG
	Intron 1-2						
2	<u>ENSE00001548747</u>	22,965,326	22,966,101	1	-	776	gtaagcaccccttcctcgggacccag.....tccaatctggcatttctccccacag GGGCCCTGGCATCOGGACAGGCATCCAAGGCCTTAAAGGAGACAGGGGAACCTGGGC CCTCTGAAACCCCGCAAGGTGGGTACCCAGGGCCAGCGCCCCCTCGAGCCCCGTG GCATCCGGGAATTAAAGGCACCAAGGCAGCCCGCAAGAAACATCAAGGACCGGAGGC CAGCCTTCTCGGCATTGGGGAAACCCCAATGGGGGCAAGTGGTGTCTTTCGACA CGGTATCACCAACAGGAACCGTACCAGAACCACTCCGGCCGATTCTCTGCATG TACCCGGGTACTACTACTTCACTTCCAGTGTGTGCCAGTGGAAATCTGCCTGTCCA TCGTCTCCTCTCAAGGGCCAGGTCCGACGCTCCCTGGGCTTCTGTACACCAACA AGGGCTCTTCCAGTGTGTACAGGGGCATGGTGTTCAGTGCAGCAGGTGACCAAG TCTGGGTGAAAGACCCCAAAAGGTCACATTTACCAGGGCTCTGAGGCCGACAGCG TCTTCAGGGCTTCTCATCTTCCCATCTGCTGAGCCAGGAGGACCCCTCCCCAC CCACCTCTTGGCTTCATGCTCCGCTGTAAATGGGGGCGTATTGCTTCAGCTGCTG AAGGAGGGGGTGGCTCTGAGACCCCGAGCTGGTGGCCCTGACACATGCTCTAAG AAGCTCGTTTCTTAGACCTCTCTGGAATAAACATCTGTGTGTCTGTCTGCTGAA catgagcttcagttgtctactcggagcattgagaggaggcctaagaataa.....
	3' downstream sequence						

8.5 Table of Top 1031 Genes with SSMD <-5 and Z-Score<1

Gene Name	Accession Number	Median Raw Values	Median Normalised Values	Median Z-Score Values	P Value	SSMD	Cell Viability	100 - CV
UBB	7314	0.067	0.107717	-3.03286	0.000348	-5.127522	10.7717	89.2283
RAN	5901	0.1	0.147275	-2.84195	0.000698	-6.875201	14.72754	85.27246
F8	2157	0.091	0.152685	-2.81585	0.000095	-3.88712	15.26846	84.73154
UBC	7316	0.103	0.165595	-2.75354	4.95E-06	-4.151425	16.55949	83.44051
TPM1	7168	0.129	0.169514	-2.73463	0.002053	-8.355112	16.95138	83.04862
GTF2H4	2968	0.145	0.190789	-2.63195	0.000255	-14.59534	19.07895	80.92105
RPL5	6125	0.132	0.219634	-2.49275	0.002321	-2.681132	21.96339	78.03661
SYMPK	8189	0.139	0.219937	-2.49129	0.000206	-43.27717	21.99367	78.00633
SRPR	6734	0.19	0.222482	-2.479	0.001476	-30.10549	22.24824	77.75176
RAD51	5888	0.154	0.223837	-2.47247	0.000318	-23.22839	22.38372	77.61628
AOX1	316	0.146	0.22956	-2.44485	0.001392	-16.04781	22.95597	77.04403
RPL10	6134	0.14	0.232945	-2.42851	0.020783	-1.501963	23.29451	76.70549
CLASP1	23332	0.151	0.233746	-2.42464	0.000487	-5.548111	23.37461	76.62539
CORO1C	23603	0.151	0.233746	-2.42464	0.005648	-2.469811	23.37461	76.62539
POLR2A	5430	0.141	0.234219	-2.42236	0.000204	-4.507618	23.42193	76.57807
SH3KBP1	30011	0.16	0.23845	-2.40194	0.002843	-8.083709	23.84501	76.15499
TRPC7	57113	0.162	0.239468	-2.39703	0.004302	-4.973624	23.94678	76.05322
KCNQ2	3785	0.164	0.240469	-2.3922	3.76E-05	-22.15481	24.04692	75.95308
CTCF	10664	0.171	0.241525	-2.3871	0.004279	-2.28184	24.15254	75.84746
SON	6651	0.154	0.243671	-2.37675	0.002948	-6.516021	24.36709	75.63291
RUNX2	860	0.125	0.246063	-2.3652	0.000699	-7.439641	24.6063	75.3937
ITGB6	3694	0.181	0.251739	-2.33781	0.001348	-4.124514	25.17385	74.82615
ZNF325	51711	0.142	0.251773	-2.33765	0.000622	-2.935426	25.17731	74.8227
TRPV2	51393	0.172	0.25425	-2.32569	0.00215	-13.14101	25.42498	74.57502
KRT10	3858	0.188	0.254398	-2.32498	0.008061	-6.273708	25.43978	74.56022
RPS29	6235	0.154	0.25624	-2.31609	0.006575	-2.423869	25.62396	74.37604
RPL37A	6168	0.157	0.261231	-2.292	6.76E-06	-3.496504	26.12313	73.87687
TREM2	54209	0.177	0.261641	-2.29003	0.001406	-15.90088	26.16408	73.83592
CCDC6	8030	0.198	0.262252	-2.28708	0.001361	-1.201236	26.22517	73.77483
TGFB1	7040	0.224	0.262295	-2.28687	0.004818	-8.258522	26.22951	73.77049
TP53	7157	0.163	0.262903	-2.28393	8.22E-05	-13.14568	26.29032	73.70968
EHF	26298	0.188	0.265537	-2.27122	0.000615	-3.336717	26.55367	73.44633
PTMA	5757	0.161	0.267887	-2.25988	0.001281	-3.174023	26.78869	73.21131
SDHD	6392	0.162	0.269551	-2.25185	0.00221	-6.48383	26.95507	73.04493
KCNAB1	7881	0.177	0.273148	-2.23449	0.002802	-7.018643	27.31481	72.68519
SLC14A1	6563	0.222	0.276119	-2.22015	0.001306	-35.22951	27.61194	72.38806
CD2BP2	10421	0.209	0.276455	-2.21853	0.000183	-5.309111	27.6455	72.3545
PDCD2	5134	0.215	0.280679	-2.19815	0.434172	-0.337277	28.06789	71.93211
LPL	4023	0.18	0.283019	-2.18685	0.000111	-8.288104	28.30189	71.69811

CIRBP	1153	0.183	0.283282	-2.18559	0.000393	-6.739147	28.32817	71.67183
SYNJ1	8867	0.189	0.283358	-2.18522	0.002049	-6.788052	28.33583	71.66417
ISGF3G	10379	0.184	0.28417	-2.1813	0.000142	-16.16719	28.41699	71.58301
RPL13A	23521	0.171	0.284526	-2.17958	0.040678	-3.055184	28.45258	71.54742
SVIL	6840	0.18	0.28481	-2.17821	0.001584	-9.297103	28.48101	71.51899
RABGEF1	27342	0.163	0.286972	-2.16778	0.004585	-5.280741	28.69718	71.30282
FOSL1	8061	0.158	0.289908	-2.15361	0.001436	-2.749821	28.99083	71.00917
CDH16	1014	0.188	0.291022	-2.14823	0.002219	-4.882238	29.10217	70.89783
GPAA1	8733	0.224	0.294737	-2.1303	0.000515	-15.39577	29.47368	70.52632
MSH6	2956	0.205	0.295815	-2.1251	0.030323	-1.993341	29.58153	70.41847
HEY1	23462	0.162	0.297248	-2.11819	0.00233	-5.387744	29.72477	70.27523
DNAJA2	10294	0.242	0.297297	-2.11795	0.006006	-5.283339	29.72973	70.27027
TADA3L	10474	0.19	0.298742	-2.11097	0.000552	-10.193	29.87421	70.12579
BCL9	607	0.222	0.298789	-2.11075	0.007779	-10.45868	29.87887	70.12113
SAMSN1	64092	0.18	0.299501	-2.10731	0.00136	-2.652299	29.95008	70.04992
FOXG1C	2292	0.229	0.301316	-2.09855	0.00032	-32.76014	30.13158	69.86842
TBL1X	6907	0.172	0.301754	-2.09644	0.000718	-11.81628	30.17544	69.82456
PKP1	5317	0.231	0.303548	-2.08778	0.003503	-6.268723	30.3548	69.6452
CSEN	30818	0.215	0.303672	-2.08718	0.002811	-5.970275	30.36723	69.63277
NR1H2	7376	0.249	0.304029	-2.08546	0.019646	-2.075779	30.40293	69.59707
UBA52	7311	0.225	0.304465	-2.08335	0.000477	-7.240774	30.44655	69.55345
CARD12	58484	0.203	0.305263	-2.0795	0.030755	-2.106255	30.52632	69.47368
DSG3	1830	0.183	0.307047	-2.0709	0.004432	-2.149895	30.7047	69.2953
ATF6	22926	0.194	0.308426	-2.06424	0.003403	-2.251869	30.84261	69.15739
RPL11	6135	0.186	0.309484	-2.05913	0.023763	-1.386672	30.94842	69.05158
TUBGCP3	10426	0.237	0.311432	-2.04973	0.000418	-23.99261	31.14323	68.85677
RELA	5970	0.266	0.311475	-2.04952	0.002196	-5.730606	31.14754	68.85246
MAFK	7975	0.202	0.311969	-2.04714	0.01632	-2.997326	31.19691	68.80309
ZNF278	23598	0.176	0.312057	-2.04672	0.009365	-1.719677	31.20567	68.79433
KCND3	3752	0.213	0.312317	-2.04546	0.00479	-3.644058	31.23167	68.76833
CDC25C	995	0.2	0.312989	-2.04222	0.000396	-7.806416	31.2989	68.7011
USP30	84749	0.201	0.313084	-2.04176	0.000372	-6.59102	31.30841	68.69159
CFL1	1072	0.203	0.314241	-2.03617	0.003631	-3.596672	31.42415	68.57585
MLLT6	4302	0.218	0.314574	-2.03457	0.006981	-3.874603	31.45743	68.54257
THBS2	7058	0.24	0.315375	-2.03071	0.002106	-5.521337	31.53745	68.46255
ALX4	60529	0.2	0.317965	-2.0182	0.002129	-2.454313	31.7965	68.2035
CORO2A	7464	0.206	0.318885	-2.01376	0.001213	-6.464036	31.88854	68.11146
MMAB	326625	0.187	0.319658	-2.01003	0.00071	-3.077119	31.96581	68.03419
MYBL2	4605	0.182	0.322124	-1.99813	0.021566	-2.14091	32.21239	67.78761
PSMD7	5713	0.189	0.323077	-1.99353	0.001059	-4.938885	32.30769	67.69231
NPTXR	23467	0.229	0.323446	-1.99175	0.000905	-7.489075	32.34463	67.65537
CDY1	9085	0.209	0.323529	-1.99135	0.00153	-3.614454	32.35294	67.64706
ABCC10	89845	0.216	0.324812	-1.98516	0.002269	-10.45389	32.4812	67.5188
GPC3	2719	0.247	0.325	-1.98425	0.000638	-9.103586	32.5	67.5
HNRPUL1	11100	0.194	0.325503	-1.98182	0.014599	-1.545291	32.55034	67.44966
TF	7018	0.206	0.325949	-1.97967	0.0011	-9.115191	32.59494	67.40506

ETV7	51513	0.231	0.326271	-1.97812	0.000817	-3.095653	32.62712	67.37288
CETN3	1070	0.211	0.326625	-1.97641	0.001618	-3.962648	32.66254	67.33746
CANX	821	0.218	0.32782	-1.97065	0.009263	-2.523781	32.78195	67.21805
CYP2D6	1565	0.25	0.328084	-1.96937	0.000476	-5.953381	32.8084	67.1916
GABRB2	2561	0.221	0.330838	-1.95608	0.030476	-1.503523	33.08383	66.91617
TRPM3	80036	0.266	0.330846	-1.95604	0.011126	-2.658029	33.08458	66.91542
SH2D3A	10045	0.222	0.330849	-1.95602	0.011035	-5.717782	33.08495	66.91505
VP552	6293	0.206	0.33119	-1.95438	0.002037	-2.467077	33.11897	66.88103
NYX	60506	0.21	0.331754	-1.95166	0.003466	-2.555991	33.17536	66.82464
DUSP9	1852	0.212	0.331768	-1.95159	0.001863	-6.083605	33.17684	66.82316
LIF	3976	0.235	0.331921	-1.95085	0.002783	-6.941046	33.19209	66.80791
IL22	50616	0.239	0.332406	-1.94851	0.005061	-2.660961	33.24061	66.75939
SIX6	4990	0.169	0.332677	-1.9472	0.002622	-9.045014	33.26772	66.73228
CYBA	1535	0.215	0.332817	-1.94653	0.00231	-3.582133	33.28173	66.71827
MYL7	58498	0.209	0.337097	-1.92588	0.003688	-5.950756	33.70968	66.29032
C8A	731	0.277	0.337805	-1.92246	0.00178	-13.66068	33.78049	66.21951
HIRIP3	8479	0.257	0.338158	-1.92075	0.001703	-6.947814	33.81579	66.18421
SIGLEC11	114132	0.214	0.338608	-1.91858	0.00045	-9.483855	33.86076	66.13924
PRKAB1	5564	0.234	0.33913	-1.91606	0.002663	-5.64749	33.91304	66.08696
TFAP2C	7022	0.216	0.339623	-1.91369	0.000457	-8.349282	33.96226	66.03774
GUCA1B	2979	0.214	0.340223	-1.91079	0.000407	-4.145536	34.02226	65.97774
PHC1	1911	0.261	0.340731	-1.90834	0.020232	-1.613617	34.07311	65.92689
SCYE1	9255	0.291	0.340749	-1.90825	0.006361	-8.15882	34.07494	65.92506
NDP52	10241	0.201	0.34359	-1.89454	0.016703	-1.427166	34.35897	65.64103
GLIS2	84662	0.205	0.34396	-1.89275	0.003958	-2.048443	34.39597	65.60403
PNMT	5409	0.208	0.344942	-1.88801	0.001336	-7.093152	34.4942	65.5058
KIF2C	11004	0.221	0.345853	-1.88362	0.003598	-8.001195	34.58529	65.41471
APBB3	10307	0.23	0.345865	-1.88356	0.004227	-8.305002	34.58647	65.41353
BAIAP3	8938	0.23	0.345865	-1.88356	0.002166	-4.474382	34.58647	65.41353
DUX1	26584	0.245	0.346045	-1.88269	0.004816	-2.525102	34.60452	65.39548
SERTAD2	9792	0.217	0.348875	-1.86904	0.0019	-2.226026	34.88746	65.11254
ATF5	22809	0.221	0.351351	-1.85708	0.00403	-3.839209	35.13514	64.86486
DSPP	1834	0.286	0.351351	-1.85708	0.037268	-1.803745	35.13514	64.86486
DNTT	1791	0.268	0.351706	-1.85537	0.001229	-6.854378	35.1706	64.8294
HIP2	3093	0.24	0.353461	-1.8469	0.003296	-3.710949	35.3461	64.6539
RABL2B	11158	0.236	0.353823	-1.84515	0.006398	-10.51479	35.38231	64.61769
LGALS3BP	3959	0.255	0.354659	-1.84112	0.015137	-2.47425	35.46592	64.53408
CPT2	1376	0.191	0.35568	-1.83619	0.00153	-23.89563	35.56797	64.43203
MAPT	4137	0.263	0.355886	-1.8352	0.010719	-2.444554	35.58863	64.41137
CASP10	843	0.203	0.35614	-1.83397	0.000839	-10.93635	35.61404	64.38596
NRCAM	4897	0.273	0.356397	-1.83273	0.003416	-106.6379	35.63969	64.36031
KRTHB1	3887	0.231	0.356481	-1.83232	0.010647	-57.18976	35.64815	64.35185
HSFY2	159119	0.277	0.356499	-1.83224	0.013981	-2.203331	35.64994	64.35006
WFS1	7466	0.211	0.35672	-1.83117	0.001997	#VALUE!	35.67202	64.32798
POLH	5429	0.215	0.357143	-1.82913	0.003702	-2.719572	35.71429	64.28571
PSMA2	5683	0.209	0.357265	-1.82854	0.038728	-1.662951	35.7265	64.2735

RAI1	10743	0.203	0.357394	-1.82792	0.007698	-9.09808	35.73944	64.26056
KNTC2	10403	0.272	0.357895	-1.8255	0.204833	-1.369495	35.78947	64.21053
CLPP	8192	0.193	0.359404	-1.81822	0.001704	-23.65794	35.94041	64.05959
FRS3	10817	0.249	0.359827	-1.81618	0.01355	-5.883521	35.98266	64.01734
K-ALPHA-1	10376	0.229	0.360063	-1.81504	0.000375	-6.289968	36.00629	63.99371
ITGB3	3690	0.259	0.360223	-1.81427	0.001886	-5.365964	36.02225	63.97775
TG	7038	0.308	0.360656	-1.81218	0.001385	-31.01243	36.06557	63.93443
SGCA	6442	0.242	0.360656	-1.81218	0.005775	-2.862697	36.06557	63.93443
PON3	5446	0.218	0.361526	-1.80798	0.002615	-3.447011	36.15257	63.84743
COL10A1	1300	0.273	0.361589	-1.80767	0.004845	-2.224802	36.15894	63.84106
GFRA4	64096	0.207	0.361888	-1.80623	0.000219	-4.833549	36.18881	63.81119
BIRC7	79444	0.241	0.362406	-1.80373	0.01429	-3.509638	36.2406	63.7594
RLN2	6019	0.311	0.364169	-1.79523	0.002212	-3.95405	36.41686	63.58314
TRAF2	7186	0.251	0.364826	-1.79206	0.002343	-7.680998	36.48256	63.51744
HERC5	51191	0.274	0.365333	-1.78961	0.005548	-13.20927	36.53333	63.46667
FOSB	2354	0.218	0.365772	-1.78749	0.000645	-2.643065	36.57718	63.42282
NDE1	54820	0.214	0.365812	-1.7873	0.001573	-2.684857	36.5812	63.4188
MEP1B	4225	0.222	0.366337	-1.78476	0.00319	-5.076928	36.63366	63.36634
PROS1	5627	0.279	0.366623	-1.78338	0.011648	-3.232609	36.66229	63.33771
GDF2	2658	0.254	0.367052	-1.78131	0.011357	-2.788065	36.7052	63.2948
SPAG1	6674	0.247	0.368107	-1.77622	0.002843	-4.517543	36.81073	63.18927
NFKBIL2	4796	0.208	0.368142	-1.77605	0.001791	-5.386924	36.81416	63.18584
ETF1	2107	0.281	0.369251	-1.7707	0.000615	-23.12719	36.9251	63.0749
CLCA2	9635	0.252	0.369501	-1.76949	0.010134	-3.666356	36.95015	63.04985
C1QA	712	0.275	0.370121	-1.7665	3.42E-05	-5.938498	37.01211	62.98789
RAPGEF6	51735	0.233	0.370429	-1.76501	5.56E-05	-3.441318	37.04293	62.95707
SSR4	6748	0.235	0.371835	-1.75823	0.038827	-1.654472	37.18354	62.81646
ANXA9	8416	0.289	0.372423	-1.75539	0.000674	-4.310971	37.24227	62.75773
JJAZ1	23512	0.205	0.372727	-1.75392	0.000275	-3.387224	37.27273	62.72727
PEX13	5194	0.3	0.373134	-1.75196	0.004883	-8.787193	37.31343	62.68657
RDX	5962	0.284	0.373193	-1.75167	0.00031	-10.26999	37.31932	62.68068
SEC14L3	266629	0.283	0.373351	-1.75091	0.001696	-20.12735	37.33509	62.66491
PPM1E	22843	0.234	0.373802	-1.74874	0.002306	-22.81646	37.38019	62.61981
DLX3	1747	0.265	0.374294	-1.74636	0.003154	-2.743712	37.42938	62.57062
CIZ1	25792	0.242	0.374613	-1.74482	0.002754	-2.951843	37.4613	62.5387
TRIP3	9326	0.307	0.374847	-1.74369	0.003897	-3.466983	37.48474	62.51526
USP18	11274	0.241	0.375389	-1.74107	0.001292	-7.534152	37.53894	62.46106
PCDHA13	56136	0.288	0.375979	-1.73823	0.004967	-2.592293	37.59791	62.40209
GATA1	2623	0.205	0.376147	-1.73742	0.000699	-2.528808	37.61468	62.38532
MAD	4084	0.244	0.376834	-1.7341	0.001039	-7.750049	37.6834	62.3166
IL2RA	3559	0.271	0.376912	-1.73373	0.029759	-3.556373	37.69124	62.30876
EGR1	1958	0.267	0.377119	-1.73273	0.002814	-4.871421	37.71186	62.28814
IFNW1	3467	0.261	0.377168	-1.73249	0.000213	-7.703439	37.71676	62.28324
FUSIP1	10772	0.225	0.377517	-1.73081	0.003947	-2.009402	37.75168	62.24832
MYH6	4624	0.262	0.378066	-1.72816	0.00465	-3.226517	37.80664	62.19336
BCL6	604	0.238	0.378378	-1.72665	0.014522	-4.138865	37.83784	62.16216

ABTB1	80325	0.254	0.381955	-1.70939	0.025914	-2.366611	38.19549	61.80451
ADAM18	8749	0.191	0.382	-1.70917	0.00541	-3.275993	38.2	61.8
POLR2G	5436	0.23	0.38206	-1.70888	0.001754	-3.942019	38.20598	61.79402
GNPDA1	10007	0.224	0.382253	-1.70795	0.010791	-5.079849	38.22526	61.77474
NUMB	8650	0.242	0.382306	-1.70769	0.004731	-2.652957	38.23065	61.76935
PHC2	1912	0.293	0.382507	-1.70673	0.002126	-4.474095	38.25065	61.74935
GSTZ1	2954	0.26	0.382916	-1.70475	0.005664	-14.95241	38.29161	61.70839
ARHGEF2	9181	0.216	0.382979	-1.70445	0.01172	-3.824147	38.29787	61.70213
PEPP2	54477	0.294	0.383812	-1.70043	0.002301	-8.138798	38.3812	61.6188
LAMR1	3921	0.272	0.384181	-1.69865	0.001382	-10.37466	38.41808	61.58192
RPS16	6217	0.231	0.384359	-1.69779	0.009409	-2.456041	38.43594	61.56406
ICAM1	3383	0.266	0.384393	-1.69762	0.001009	-5.781329	38.43931	61.56069
RRM2	6241	0.214	0.384892	-1.69522	0.003659	-6.612658	38.48921	61.51079
SRCAP	10847	0.239	0.385484	-1.69236	0.005441	-3.824227	38.54839	61.45161
WNT8B	7479	0.261	0.385809	-1.69079	0.005291	-9.008995	38.58093	61.41907
NR1H3	10062	0.316	0.385836	-1.69066	0.027307	-1.938551	38.58364	61.41636
GAB2	9846	0.23	0.385906	-1.69032	0.001708	-3.529475	38.5906	61.4094
ALX3	257	0.243	0.386328	-1.68829	0.001874	-2.680418	38.63275	61.36725
IL9R	3581	0.278	0.386648	-1.68674	0.004014	-5.330966	38.66481	61.33519
CIB1	10519	0.248	0.388106	-1.6797	0.001049	-5.743023	38.81064	61.18936
LISCH7	51599	0.283	0.388736	-1.67666	0.003618	-5.675129	38.87363	61.12637
ZBTB10	65986	0.234	0.389351	-1.6737	0.066244	-2.166035	38.93511	61.06489
KIF1C	10749	0.249	0.389671	-1.67215	0.005401	-6.245861	38.96714	61.03286
TKTL1	8277	0.26	0.389805	-1.6715	0.003947	-3.99259	38.98051	61.01949
PERP	64065	0.269	0.389855	-1.67126	0.001858	-2.101148	38.98551	61.01449
FBXL11	22992	0.293	0.390667	-1.66735	0.001492	-10.91748	39.06667	60.93333
TRIM28	10155	0.293	0.390667	-1.66735	0.000123	-5.428506	39.06667	60.93333
ATF7IP	55729	0.26	0.390977	-1.66585	0.00653	-6.126194	39.09774	60.90226
CES1	1066	0.21	0.391061	-1.66544	0.000106	-11.15881	39.10615	60.89385
SAG	6295	0.246	0.391097	-1.66527	0.001696	-2.603402	39.1097	60.8903
TLR2	7097	0.334	0.391101	-1.66525	0.003457	-18.99032	39.11007	60.88993
PROK1	84432	0.334	0.391101	-1.66525	0.007939	-7.373039	39.11007	60.88993
CAPN2	824	0.223	0.391228	-1.66464	0.001758	-7.446653	39.12281	60.87719
TAT	6898	0.261	0.391304	-1.66427	0.006117	-12.458	39.13043	60.86957
KCNJ8	3764	0.268	0.392962	-1.65627	0.00124	-11.0729	39.29619	60.70381
CD5	921	0.294	0.393048	-1.65585	0.000544	-4.419852	39.30481	60.69519
EMX1	2016	0.279	0.394068	-1.65093	0.002104	-2.621867	39.40678	60.59322
HF1	3075	0.3	0.394737	-1.6477	0.008287	-3.960671	39.47368	60.52632
EREG	2069	0.273	0.39508	-1.64605	0.003228	-3.653412	39.50796	60.49204
ABCG5	64240	0.263	0.395489	-1.64408	0.007258	-4.581886	39.54887	60.45113
HSPB2	3316	0.249	0.395866	-1.64225	0.013377	-2.327831	39.58665	60.41335
TNFRSF8	943	0.268	0.396157	-1.64085	0.003767	-13.27703	39.61567	60.38433
CUGBP2	10659	0.256	0.396285	-1.64023	0.018772	-2.576702	39.62848	60.37152
CREM	1390	0.281	0.396893	-1.6373	0.005715	-2.060113	39.68927	60.31073
IRF2	3660	0.257	0.396911	-1.63721	0.016046	-2.881617	39.69112	60.30888
BAIAP1	9223	0.264	0.396992	-1.63682	0.002131	-15.84832	39.69925	60.30075

COL9A2	1298	0.3	0.397351	-1.63509	0.018106	-1.725688	39.7351	60.2649
SRD5A1	6715	0.221	0.397482	-1.63446	0.002339	-5.844613	39.7482	60.2518
MPRG	54852	0.233	0.398291	-1.63055	0.004708	-2.624932	39.82906	60.17094
LDB1	8861	0.258	0.398456	-1.62976	0.00262	-12.18055	39.84556	60.15444
TNNI2	7136	0.248	0.398714	-1.62851	0.00044	-3.143866	39.87138	60.12862
RHD	6007	0.24	0.399334	-1.62552	0.001075	-3.409246	39.93344	60.06656
HRK	8739	0.242	0.39934	-1.62549	0.003152	-3.871104	39.93399	60.06601
CSF3	1440	0.299	0.399733	-1.62359	0.002045	-4.198297	39.97326	60.02674
HOXC5	3222	0.259	0.4	-1.6223	0.000658	-14.27077	40	60
PTCRA	171558	0.234	0.4	-1.6223	0.001048	-6.246468	40	60
PDLIM1	9124	0.307	0.400783	-1.61852	0.003872	-2.614511	40.07833	59.92167
C9ORF125	84302	0.243	0.40099	-1.61753	0.002739	-3.404109	40.09901	59.90099
ATP5C1	509	0.298	0.401077	-1.61711	0.001675	-4.621627	40.10767	59.89233
DDIT3	1649	0.284	0.40113	-1.61685	0.000825	-2.277435	40.11299	59.88701
PSMD8	5714	0.235	0.401709	-1.61405	0.007838	-18.62681	40.17094	59.82906
SLC6A2	6530	0.323	0.401741	-1.6139	0.001197	-6.0061	40.17413	59.82587
TGFB2	7042	0.229	0.401754	-1.61384	0.000919	-11.44681	40.17544	59.82456
MNDA	4332	0.279	0.402597	-1.60977	4.94E-05	-6.678804	40.25974	59.74026
GYPA	2993	0.306	0.402632	-1.6096	0.013215	-2.806918	40.26316	59.73684
HAND2	9464	0.306	0.402632	-1.6096	0.009954	-3.136813	40.26316	59.73684
IFNA7	3444	0.244	0.40264	-1.60956	0.004079	-8.400837	40.26403	59.73597
FLJ20331	55631	0.313	0.402831	-1.60864	0.003405	-3.780361	40.28314	59.71686
CRISP1	167	0.332	0.403893	-1.60352	0.007283	-7.411065	40.38929	59.61071
NTF3	4908	0.345	0.403981	-1.60309	0.140019	-1.843781	40.39813	59.60187
MSH4	4438	0.28	0.40404	-1.60281	0.000859	-7.708515	40.40404	59.59596
FMR1	2332	0.241	0.404362	-1.60125	0.002086	-3.152762	40.43624	59.56376
GMPR2	51292	0.237	0.404437	-1.60089	0.007458	-7.296794	40.44369	59.55631
TNA	7123	0.256	0.405063	-1.59787	0.003567	-7.276493	40.50633	59.49367
ASH1L	55870	0.255	0.405405	-1.59622	0.027452	-6.670712	40.54054	59.45946
DDX11	1663	0.309	0.405512	-1.5957	0.003409	-5.575408	40.55118	59.44882
PRPF8	10594	0.232	0.408451	-1.58152	0.008963	-8.333599	40.84507	59.15493
CCNK	8812	0.335	0.408537	-1.58111	0.000433	-11.68259	40.85366	59.14634
IL11	3589	0.285	0.41185	-1.56512	0.000163	-5.555041	41.18497	58.81503
ELK4	2005	0.295	0.416667	-1.54187	0.005058	-5.300207	41.66667	58.33333
KLF1	10661	0.27	0.416988	-1.54032	0.004499	-7.038929	41.69884	58.30116
ZNF589	51385	0.264	0.417722	-1.53678	0.001759	-5.810273	41.77215	58.22785
ELF4	2000	0.297	0.419492	-1.52824	0.036658	-5.914265	41.94915	58.05085
HSF1	3297	0.272	0.420077	-1.52541	0.003605	-6.086172	42.00772	57.99228
SPAP1	79368	0.285	0.421286	-1.51958	0.000747	-5.743646	42.1286	57.8714
CNOT2	4848	0.321	0.421813	-1.51703	0.000291	-15.05361	42.18134	57.81866
CBX1	10951	0.346	0.421951	-1.51637	0.000455	-13.06849	42.19512	57.80488
M160	283316	0.299	0.422316	-1.51461	0.000422	-7.942475	42.23164	57.76836
CSAD	51380	0.227	0.422719	-1.51266	0.000286	-9.595474	42.27188	57.72812
TEP1	7011	0.282	0.422789	-1.51233	0.005277	-11.24143	42.27886	57.72114
RSC1A1	6248	0.341	0.424129	-1.50586	0.004231	-12.56229	42.41294	57.58706
ATP7A	538	0.27	0.424528	-1.50393	0.002333	-22.87496	42.45283	57.54717

SLC25A4	291	0.285	0.424739	-1.50291	0.001533	-5.922293	42.47392	57.52608
NKIRAS1	28512	0.267	0.425159	-1.50089	0.004818	-54.88635	42.51592	57.48408
HCNGP	29115	0.324	0.426316	-1.4953	0.000607	-23.73746	42.63158	57.36842
NHP2L1	4809	0.325	0.42707	-1.49167	0.000173	-25.38912	42.70696	57.29304
KSR	8844	0.269	0.428344	-1.48552	0.003412	-9.210695	42.83439	57.16561
PROP1	5626	0.218	0.429134	-1.4817	0.001653	-8.266277	42.91339	57.08661
IL17	3605	0.297	0.429191	-1.48143	0.000194	-5.108018	42.91908	57.08092
GJB1	2705	0.294	0.431085	-1.47229	0.004627	-5.559818	43.1085	56.8915
APOA2	336	0.333	0.431347	-1.47102	0.005219	-6.946153	43.13472	56.86529
ALDH1A3	220	0.216	0.432	-1.46787	0.000529	-7.254402	43.2	56.8
COMT	1312	0.33	0.433071	-1.4627	0.005372	-5.116174	43.30709	56.69291
TEAD3	7005	0.276	0.433962	-1.4584	0.000415	-6.419634	43.39623	56.60377
ENTPD5	957	0.331	0.434383	-1.45637	0.005148	-5.11014	43.43832	56.56168
OTUB1	55611	0.279	0.434579	-1.45542	0.003857	-12.0847	43.45794	56.54206
MTM1	4534	0.278	0.435055	-1.45313	0.000127	-21.0981	43.50548	56.49452
RAB3B	5865	0.296	0.435935	-1.44888	0.00821	-23.53897	43.59352	56.40648
TNFRSF12 A	51330	0.373	0.436768	-1.44486	0.002201	-18.65459	43.67682	56.32319
GLI4	2738	0.261	0.437919	-1.43931	0.003155	-5.37348	43.79195	56.20805
CTSW	1521	0.236	0.439479	-1.43178	0.000827	-6.625392	43.94786	56.05214
ATP6V1B1	525	0.28	0.440252	-1.42805	0.012753	-6.864567	44.02516	55.97484
CPN2	1370	0.362	0.440389	-1.42739	0.003931	-6.897373	44.03893	55.96107
LOC25457 1	254571	0.321	0.440934	-1.42476	0.002568	-9.627952	44.09341	55.90659
MMP24	10893	0.3	0.441826	-1.42045	0.018526	-5.686272	44.18262	55.81738
PLAGL1	5325	0.251	0.441901	-1.42009	0.001042	-12.18155	44.19014	55.80986
LOC11497 1	114971	0.283	0.442879	-1.41537	0.000185	-12.27304	44.28795	55.71205
BCL11A	53335	0.295	0.443609	-1.41185	0.001982	-12.06404	44.3609	55.6391
PPP1R3A	5506	0.276	0.445161	-1.40436	0.001553	-5.496815	44.51613	55.48387
CDC25B	994	0.285	0.446009	-1.40026	0.001171	-6.000958	44.60094	55.39906
PIGR	5284	0.359	0.446517	-1.39781	0.000352	-10.78298	44.65174	55.34826
WFDC1	58189	0.277	0.446774	-1.39657	0.003259	-5.530527	44.67742	55.32258
MSLN	10232	0.311	0.448773	-1.38692	0.00528	-5.714012	44.87734	55.12266
PRND	23627	0.31	0.449275	-1.3845	0.014412	-6.756715	44.92754	55.07246
THBD	7056	0.387	0.453162	-1.36575	0.004767	-6.96977	45.31616	54.68384
TCAP	8557	0.336	0.454668	-1.35847	0.005389	-5.425991	45.46685	54.53315
SCNN1A	6337	0.366	0.455224	-1.35579	0.003111	-7.860782	45.52239	54.47761
F7	2155	0.347	0.455381	-1.35504	0.011192	-15.20068	45.53806	54.46194
TAX1BP1	8887	0.288	0.455696	-1.35351	0.00016	-15.2022	45.56962	54.43038
INPP5F	22876	0.296	0.45679	-1.34824	0.018492	-21.16127	45.67901	54.32099
TMC1	117531	0.261	0.457895	-1.3429	0.001777	-7.530266	45.78947	54.21053
SGSH	6448	0.255	0.458633	-1.33934	5.89E-05	-7.803036	45.86331	54.13669
S100A14	57402	0.261	0.459507	-1.33512	0.003234	-17.83306	45.9507	54.0493
SPINK5	11005	0.285	0.459677	-1.3343	0.001126	-16.03667	45.96774	54.03226
ADAMTS2 O	80070	0.278	0.460265	-1.33147	0.004489	-7.602235	46.02649	53.97351
MARCO	8685	0.326	0.460452	-1.33056	0.00116	-10.20242	46.0452	53.9548
RANBP2L1	84220	0.263	0.463028	-1.31813	0.000152	-5.460371	46.30282	53.69718

RAP1A	5906	0.309	0.463268	-1.31697	0.006651	-11.87671	46.32684	53.67316
LILRB4	11006	0.328	0.463277	-1.31693	0.004537	-5.567216	46.32768	53.67232
ADAMTS1 7	170691	0.28	0.463576	-1.31549	0.005029	-7.037039	46.35762	53.64238
FDFT1	2222	0.354	0.464567	-1.3107	0.005983	-24.42612	46.45669	53.54331
MS4A1	931	0.329	0.464689	-1.31011	0.001394	-7.311623	46.46893	53.53107
CENPA	1058	0.301	0.465944	-1.30406	0.012452	-5.025626	46.59443	53.40557
DOLPP1	57171	0.298	0.466354	-1.30208	0.000948	-6.142119	46.63537	53.36463
RFC4	5984	0.265	0.466549	-1.30114	0.010216	-8.316645	46.65493	53.34507
UCN	7349	0.312	0.467066	-1.29864	0.000673	-7.094989	46.70659	53.29341
MYF6	4618	0.264	0.467257	-1.29772	0.006573	-7.172679	46.72566	53.27434
PNUTL1	5413	0.298	0.468553	-1.29147	0.000387	-7.243294	46.85535	53.14465
M96	22823	0.325	0.468975	-1.28943	0.009834	-7.766811	46.89755	53.10245
BCS1L	617	0.312	0.469173	-1.28848	0.001663	-6.231019	46.91729	53.08271
GBA	2629	0.275	0.469283	-1.28794	0.001534	-22.7832	46.92833	53.07167
C1S	716	0.269	0.47193	-1.27517	0.000276	-16.79528	47.19298	52.80702
HBE1	3046	0.359	0.472368	-1.27305	0.001847	-5.761381	47.23684	52.76316
RAB5A	5868	0.321	0.472754	-1.27119	0.0054	-9.528493	47.27541	52.7246
FLJ20343	54902	0.297	0.47293	-1.27034	0.005865	-12.78517	47.29299	52.70701
IL13	3596	0.307	0.473765	-1.26631	0.016907	-37.40173	47.37654	52.62346
M6PRBP1	10226	0.321	0.474501	-1.26276	0.005214	-7.443678	47.45011	52.54989
GPX1	2876	0.361	0.475	-1.26035	0.000147	-12.76644	47.5	52.5
CALML5	51806	0.316	0.475188	-1.25945	0.008554	-5.535264	47.5188	52.4812
GRB10	2887	0.273	0.477273	-1.24939	0.00022	-5.399189	47.72727	52.27273
BRD4	23476	0.355	0.477793	-1.24688	0.003703	-11.56311	47.77927	52.22073
SPIB	6689	0.304	0.477987	-1.24594	0.004308	-38.81624	47.79874	52.20126
RAP2A	5911	0.319	0.478261	-1.24462	0.004095	-6.673225	47.82609	52.17391
DEDD	9191	0.309	0.478328	-1.24429	0.000129	-5.174026	47.83282	52.16718
GTF3A	2971	0.261	0.478899	-1.24154	0.012319	-5.180925	47.88991	52.11009
HIG2	29923	0.365	0.480263	-1.23495	0.001853	-6.73704	48.02632	51.97368
TUBB5	10382	0.306	0.481132	-1.23076	0.013461	-7.690171	48.11321	51.88679
TAF12	6883	0.306	0.481132	-1.23076	0.000559	-6.501122	48.11321	51.88679
S100A9	6280	0.411	0.481265	-1.23012	0.005495	-23.61579	48.12646	51.87354
TFR2	7036	0.268	0.482014	-1.2265	0.001477	-19.95783	48.20144	51.79856
HDAC4	9759	0.31	0.482866	-1.22239	0.002923	-6.755265	48.2866	51.7134
GRB7	2886	0.277	0.484266	-1.21564	0.00151	-9.04069	48.42657	51.57343
NSDHL	50814	0.284	0.484642	-1.21382	0.001132	-15.69702	48.46416	51.53584
HMGCR	3156	0.284	0.484642	-1.21382	0.002111	-14.41537	48.46416	51.53584
CABC1	56997	0.398	0.485366	-1.21033	0.001497	-18.51401	48.53659	51.46341
NEDD4L	23327	0.334	0.485465	-1.20985	0.01066	-5.189818	48.54651	51.45349
ADAMTS1 2	81792	0.309	0.485849	-1.208	0.018594	-5.479201	48.58491	51.41509
CLDN5	7122	0.367	0.486093	-1.20682	0.01501	-6.436623	48.60927	51.39073
SCN4A	6329	0.391	0.486318	-1.20573	0.000819	-13.7207	48.63184	51.36816
ATF4	468	0.307	0.488076	-1.19725	0.019071	-18.5073	48.80763	51.19237
ADH1B	125	0.37	0.488127	-1.19701	0.004762	-5.086589	48.81266	51.18734
NCR3	259197	0.346	0.488701	-1.19424	0.003423	-6.452887	48.87006	51.12994

DOC-1R	10263	0.398	0.488943	-1.19306	0.003373	-12.98878	48.89435	51.10565
RBBP6	5930	0.367	0.489333	-1.19118	0.001885	-8.269227	48.93333	51.06667
CRYBB1	1414	0.403	0.490268	-1.18667	0.004262	-12.79227	49.02676	50.97324
HBA2	3040	0.373	0.490789	-1.18415	0.001034	-8.020971	49.07895	50.92105
APOF	319	0.382	0.492268	-1.17702	0.005807	-5.437712	49.2268	50.7732
DCLRE1A	9937	0.376	0.493438	-1.17137	0.006393	-11.63618	49.34383	50.65617
LTF	4057	0.31	0.493631	-1.17044	0.00056	-5.646854	49.36306	50.63694
TCF20	6942	0.314	0.493711	-1.17006	0.00059	-5.280746	49.37107	50.62893
KAL1	3730	0.376	0.494087	-1.16824	0.003108	-7.590036	49.40867	50.59133
GP9	2815	0.295	0.494966	-1.164	0.008152	-6.715152	49.49664	50.50336
TGFBI	7045	0.423	0.495316	-1.16231	0.006397	-5.016988	49.53162	50.46838
NINJ1	4814	0.344	0.496392	-1.15711	0.001089	-8.996825	49.63925	50.36075
CHML	1122	0.308	0.496774	-1.15527	0.001739	-12.9505	49.67742	50.32258
ATP2C1	27032	0.316	0.496855	-1.15488	0.00829	-13.37863	49.68553	50.31447
IFNA6	3443	0.344	0.49711	-1.15365	0.001519	-6.292866	49.71098	50.28902
GGCX	2677	0.292	0.498294	-1.14794	0.006255	-5.631357	49.82935	50.17065
MARCKS	4082	0.364	0.5	-1.1397	0.002338	-17.36764	50	50
MBL2	4153	0.347	0.500722	-1.13622	0.002083	-10.72338	50.07215	49.92785
MLC1	23209	0.342	0.501466	-1.13263	0.00053	-11.4758	50.14663	49.85337
ABP1	26	0.303	0.501656	-1.13171	0.004079	-20.49804	50.16556	49.83444
CCT8	10694	0.286	0.501754	-1.13124	0.004367	-5.060388	50.17544	49.82456
PDPR	55066	0.303	0.502488	-1.1277	0.012227	-11.08867	50.24876	49.75124
DGKH	160851	0.27	0.502793	-1.12622	0.000374	-7.70716	50.27933	49.72067
HLA-DQB1	3119	0.288	0.503497	-1.12283	0.001451	-6.896314	50.34965	49.65035
POMC	5443	0.43	0.503513	-1.12275	0.005833	-10.92941	50.35129	49.64871
RUVBL1	8607	0.336	0.503748	-1.12162	0.010659	-7.152997	50.37481	49.62519
KIF5B	3799	0.322	0.503912	-1.12082	0.000751	-9.660833	50.39124	49.60876
DAG1	1605	0.377	0.504011	-1.12035	0.002789	-8.077231	50.40107	49.59893
PLAT	5327	0.304	0.504146	-1.1197	0.005267	-9.348041	50.41459	49.58541
OGG1	4968	0.304	0.504983	-1.11565	0.007189	-6.484193	50.49834	49.50166
PCDHAC1	56135	0.387	0.505222	-1.1145	0.005252	-5.871125	50.52219	49.47781
ANAPC2	29882	0.379	0.505333	-1.11397	0.003233	-7.383472	50.53333	49.46667
USP37	57695	0.325	0.506231	-1.10964	0.010777	-9.581975	50.62305	49.37695
ALS2CR4	65062	0.393	0.506443	-1.10861	0.022228	-5.147215	50.64433	49.35567
SERPINI1	5274	0.314	0.506452	-1.10857	0.001701	-10.27243	50.64516	49.35484
CYP2C8	1558	0.272	0.506518	-1.10825	0.00307	-16.97058	50.65177	49.34823
GRIA3	2892	0.29	0.506993	-1.10596	0.003542	-8.409886	50.6993	49.3007
CTGF	1490	0.328	0.50774	-1.10235	0.011769	-5.670157	50.77399	49.22601
LMNB1	4001	0.37	0.508242	-1.09993	0.004691	-7.978459	50.82418	49.17582
PDHA2	5161	0.307	0.509121	-1.09569	0.010687	-7.098947	50.91211	49.08789
CTRL	1506	0.274	0.510242	-1.09028	0.003112	-17.66775	51.02421	48.97579
CGI-14	51005	0.274	0.510242	-1.09028	0.004359	-6.533568	51.02421	48.97579
HCBP6	65991	0.388	0.510526	-1.0889	0.000581	-8.414456	51.05263	48.94737
WNT10A	80326	0.346	0.511456	-1.08442	0.002428	-5.167821	51.1456	48.8544
SH3MD1	9644	0.344	0.512668	-1.07857	0.00128	-6.172438	51.26677	48.73323
GRIN2C	2905	0.39	0.513158	-1.0762	0.0008	-22.17868	51.31579	48.68421

CUBN	8029	0.343	0.513473	-1.07468	0.000603	-5.726982	51.34731	48.65269
RAB5C	5878	0.349	0.513991	-1.07218	0.009103	-5.15844	51.39912	48.60088
PECR	55825	0.31	0.514096	-1.07168	0.013235	-17.15556	51.40962	48.59038
TCF7	6932	0.327	0.514151	-1.07141	0.003074	-6.653557	51.41509	48.58491
SMC4L1	10051	0.325	0.514241	-1.07098	2.24E-05	-23.34684	51.42405	48.57595
LDB2	9079	0.333	0.514286	-1.07076	0.006566	-6.279831	51.42857	48.57143
DIA1	1727	0.299	0.515517	-1.06482	0.000687	-10.36297	51.55172	48.44828
MCOLN3	55283	0.352	0.516129	-1.06187	0.000157	-16.49764	51.6129	48.3871
CLCN1	1180	0.352	0.516129	-1.06187	0.003844	-8.301841	51.6129	48.3871
SPG3A	51062	0.345	0.517241	-1.0565	0.008694	-7.834248	51.72414	48.27586
SPI1	6688	0.329	0.517296	-1.05624	0.001782	-8.436388	51.72956	48.27044
TACSTD2	4070	0.35	0.517369	-1.05588	0.003791	-10.40281	51.73688	48.26312
RPS4X	6191	0.311	0.517471	-1.05539	0.025679	-13.47698	51.74709	48.25291
SH3GL3	6457	0.348	0.518629	-1.0498	0.010492	-9.000167	51.86289	48.13711
RABL2A	11159	0.346	0.518741	-1.04926	0.003733	-6.925126	51.87406	48.12594
POM121L1	25812	0.358	0.518841	-1.04878	0.033401	-9.105417	51.88406	48.11594
CHGA	1113	0.336	0.520124	-1.04259	0.005393	-13.69668	52.01238	47.98762
TAF5L	27097	0.331	0.52044	-1.04106	0.005606	-55.87107	52.04403	47.95597
TUBB	7280	0.331	0.52044	-1.04106	0.002892	-9.048386	52.04403	47.95597
TBRG4	9238	0.428	0.520681	-1.0399	0.002493	-14.21758	52.06813	47.93187
SH2D3C	10044	0.323	0.520968	-1.03851	0.004538	-5.888045	52.09677	47.90323
HIC1	3090	0.284	0.521101	-1.03787	0.016719	-7.693494	52.11009	47.88991
TRIM32	22954	0.391	0.521333	-1.03675	0.002455	-13.97876	52.13333	47.86667
FBXO4	26272	0.391	0.521333	-1.03675	0.016337	-5.805308	52.13333	47.86667
BDNF	627	0.39	0.52139	-1.03647	0.011954	-11.00484	52.13904	47.86096
TIF1	8805	0.359	0.521802	-1.03449	0.000232	-8.728732	52.18023	47.81977
WNT5B	81029	0.353	0.521803	-1.03448	0.007804	-8.780129	52.18034	47.81966
KRTHA5	3886	0.386	0.522327	-1.03195	0.001043	-6.128438	52.23275	47.76725
SLC10A2	6555	0.42	0.522388	-1.03166	0.000275	-5.210492	52.23881	47.76119
CTSB	1508	0.281	0.523277	-1.02737	0.006021	-8.679206	52.32775	47.67225
IL22RA1	58985	0.377	0.524339	-1.02224	0.009268	-6.834361	52.43394	47.56606
PHLDA3	23612	0.362	0.524638	-1.0208	0.025609	-6.916841	52.46377	47.53623
CTBP1	1487	0.4	0.524934	-1.01937	0.005786	-6.376819	52.49344	47.50656
DBR1	51163	0.282	0.52514	-1.01838	0.000931	-5.151931	52.51397	47.48603
HDAC5	10014	0.357	0.525773	-1.01532	0.00795	-9.05514	52.57732	47.42268
BCAR1	9564	0.391	0.526245	-1.01305	0.005513	-8.920723	52.6245	47.3755
ZFP161	7541	0.297	0.526596	-1.01135	0.010373	-6.561862	52.65957	47.34043
MIR	220972	0.395	0.526667	-1.01101	0.012424	-6.656809	52.66667	47.33333
GSTP1	2950	0.401	0.527632	-1.00635	0.004704	-5.15611	52.76316	47.23684
HDAC7A	51564	0.359	0.528719	-1.00111	0.006174	-13.90533	52.87187	47.12813
CPO	130749	0.284	0.528864	-1.00041	0.000866	-14.3729	52.88641	47.11359
USH2A	7399	0.391	0.529093	-0.9993	0.003352	-5.832822	52.90934	47.09066
TDGF1	6997	0.452	0.529274	-0.99843	0.004629	-11.29916	52.9274	47.0726
PLAUR	5329	0.375	0.529661	-0.99656	0.00409	-28.49901	52.9661	47.0339
SOCS2	8835	0.453	0.530445	-0.99278	0.010708	-5.837934	53.0445	46.9555
CCNT2	905	0.435	0.530488	-0.99257	0.002877	-12.99141	53.04878	46.95122

USP36	57602	0.341	0.531153	-0.98936	0.005594	-5.63472	53.11526	46.88474
ADH7	131	0.338	0.531447	-0.98794	0.004749	-11.64338	53.14465	46.85535
TBCD	6904	0.303	0.531579	-0.9873	0.00386	-5.185727	53.15789	46.84211
MTMR8	55613	0.333	0.531949	-0.98552	0.016365	-6.319808	53.19489	46.80511
WNT8A	7478	0.36	0.532151	-0.98455	0.002079	-24.59084	53.21508	46.78492
ATP8A1	10396	0.339	0.533019	-0.98036	0.000912	-6.788286	53.30189	46.69811
RYR3	6263	0.437	0.533578	-0.97766	0.005304	-6.470513	53.35775	46.64225
ZIC2	7546	0.301	0.533688	-0.97713	0.010624	-5.350237	53.36879	46.63121
CFLAR	8837	0.331	0.533871	-0.97624	0.004866	-6.440282	53.3871	46.6129
GABARAPL1	23710	0.415	0.534106	-0.97511	0.003082	-20.44677	53.41055	46.58945
SERPINA4	5267	0.359	0.535022	-0.97069	0.001107	-5.525673	53.50224	46.49776
RECQL4	9401	0.357	0.535232	-0.96967	0.004898	-8.921736	53.52324	46.47676
DRB1	129831	0.436	0.535627	-0.96777	0.001737	-44.29996	53.56265	46.43735
NFKB1	4790	0.303	0.536283	-0.9646	0.01009	-23.85046	53.62832	46.37168
DAZ3	57054	0.441	0.536496	-0.96357	0.00255	-6.720577	53.64964	46.35037
CCNE2	9134	0.44	0.536585	-0.96314	0.006554	-7.853193	53.65854	46.34146
LAMC3	10319	0.409	0.537451	-0.95897	0.001953	-6.093123	53.74507	46.25493
MMP17	4326	0.365	0.537555	-0.95846	0.012678	-7.266291	53.75552	46.24448
CSHL1	1444	0.403	0.53877	-0.9526	0.012411	-15.4714	53.87701	46.12299
SCN1B	6324	0.434	0.539801	-0.94763	0.008262	-7.178531	53.9801	46.0199
CYLD	1540	0.347	0.540498	-0.94426	0.006971	-7.906451	54.04984	45.95016
S100A5	6276	0.325	0.540765	-0.94297	0.030357	-18.53871	54.07654	45.92346
TAF6	6878	0.344	0.540881	-0.94242	0.012117	-8.457708	54.08805	45.91195
HMOX2	3163	0.317	0.540956	-0.94205	0.006677	-11.64503	54.09556	45.90444
DAZ	1617	0.35	0.541796	-0.938	0.003859	-5.174908	54.17957	45.82043
CCL26	10344	0.406	0.542781	-0.93324	0.004	-8.267724	54.27807	45.72193
PPAN	56342	0.327	0.543189	-0.93127	0.007365	-5.465855	54.31894	45.68106
GABRG3	2567	0.363	0.543413	-0.93019	0.002825	-5.688504	54.34132	45.65868
DTX1	1840	0.374	0.543605	-0.92927	0.000318	-6.455547	54.36047	45.63953
CTSC	1075	0.292	0.543762	-0.92851	0.004882	-11.12898	54.37616	45.62384
COX11	1353	0.346	0.544025	-0.92724	0.007757	-20.27177	54.40252	45.59748
TBX21	30009	0.346	0.544025	-0.92724	0.00877	-9.389673	54.40252	45.59748
WNT4	54361	0.369	0.545455	-0.92034	0.000362	-6.667589	54.54545	45.45455
MYH13	8735	0.378	0.545455	-0.92034	0.007139	-19.81408	54.54545	45.45455
TIAF1	9220	0.311	0.545614	-0.91957	0.003383	-5.937553	54.5614	45.4386
CROT	54677	0.293	0.545624	-0.91952	0.009795	-5.708293	54.56238	45.43762
B4GALT1	2683	0.273	0.546	-0.91771	0.005764	-9.792719	54.6	45.4
ADAMTS10	81794	0.33	0.546358	-0.91598	0.002934	-5.032089	54.63576	45.36424
KCNQ5	56479	0.356	0.549383	-0.90138	0.03474	-8.426029	54.93827	45.06173
SGEF	26084	0.451	0.55	-0.8984	0.000671	-9.750193	55	45
TCF7L1	83439	0.35	0.550314	-0.89689	0.015037	-5.40913	55.03145	44.96855
FBXO7	25793	0.413	0.550667	-0.89519	0.00605	-18.95631	55.06667	44.93333
C6ORF133	23304	0.413	0.550667	-0.89519	0.00637	-10.07684	55.06667	44.93333
ITGA8	8516	0.396	0.550765	-0.89471	0.015133	-13.1926	55.0765	44.9235
IFITM1	8519	0.346	0.550955	-0.89379	0.004577	-9.791407	55.09554	44.90446

TLR9	54106	0.373	0.551367	-0.89181	0.004272	-15.56812	55.13673	44.86327
PLOD	5351	0.333	0.552239	-0.8876	0.009053	-12.91288	55.22388	44.77612
NCR1	9437	0.391	0.55226	-0.8875	0.009635	-5.975878	55.22599	44.77401
SGCG	6445	0.371	0.552906	-0.88438	0.007624	-39.25388	55.29061	44.70939
SCARB1	949	0.392	0.553672	-0.88068	0.001648	-5.846641	55.36723	44.63277
EP300	2033	0.376	0.553756	-0.88028	0.013601	-15.43008	55.37555	44.62445
SIGLECL1	89858	0.35	0.553797	-0.88008	0.001819	-5.42544	55.37975	44.62025
DUSP11	8446	0.354	0.553991	-0.87915	0.003587	-10.58299	55.39906	44.60094
BENE	7851	0.412	0.554509	-0.87665	0.016196	-9.493653	55.45087	44.54913
HGFAC	3083	0.325	0.554608	-0.87617	0.001831	-12.44202	55.46075	44.53925
DNCI1	1780	0.344	0.554839	-0.87505	0.004577	-6.31571	55.48387	44.51613
PTGS1	5742	0.377	0.555228	-0.87317	0.013317	-12.64138	55.52283	44.47717
GMEB1	10691	0.303	0.555963	-0.86963	0.044595	-6.101347	55.59633	44.40367
DNMT3A	1788	0.424	0.55643	-0.86737	0.013738	-10.81173	55.64304	44.35696
ZNF124	7678	0.314	0.556738	-0.86589	0.039854	-5.011665	55.67376	44.32624
ECHS1	1892	0.323	0.556897	-0.86512	0.007676	-5.624354	55.68966	44.31034
THBS4	7060	0.424	0.557162	-0.86384	0.001523	-6.207847	55.71616	44.28384
SHH	6469	0.31	0.557554	-0.86195	0.003047	-15.71595	55.7554	44.2446
FBXO21	23014	0.425	0.557743	-0.86104	0.00951	-8.945255	55.77428	44.22572
TMP21	10972	0.423	0.558047	-0.85957	0.009381	-5.683463	55.80475	44.19525
GSPT1	2935	0.425	0.559211	-0.85395	0.005176	-5.24284	55.92105	44.07895
NXF	266743	0.396	0.559322	-0.85342	0.004886	-7.639441	55.9322	44.0678
CAPNS1	826	0.319	0.559649	-0.85184	0.001036	-9.383423	55.96491	44.03509
DMWD	1762	0.456	0.560197	-0.8492	0.007153	-6.519879	56.01966	43.98034
ANXA13	312	0.373	0.560902	-0.84579	0.000616	-5.14953	56.09023	43.90977
UBR1	197131	0.386	0.561047	-0.84509	0.001449	-9.903146	56.10465	43.89535
ZNF482	10773	0.317	0.562057	-0.84022	0.010657	-5.612665	56.20567	43.79433
GP6	51206	0.389	0.562139	-0.83982	0.005797	-5.547583	56.21387	43.78613
PLXNA4	57671	0.388	0.562319	-0.83895	0.048527	-7.568953	56.23188	43.76812
76P	27229	0.425	0.562914	-0.83608	0.003014	-7.189409	56.29139	43.70861
ACHE	43	0.34	0.562914	-0.83608	0.00789	-11.04083	56.29139	43.70861
PPP2R5A	5525	0.353	0.563898	-0.83133	0.005203	-17.99138	56.38978	43.61022
UBE2E1	7324	0.314	0.564748	-0.82723	0.004209	-13.12749	56.47482	43.52518
BIRC1	4671	0.322	0.564912	-0.82644	0.001066	-9.198661	56.49123	43.50877
SOS2	6655	0.351	0.566129	-0.82057	0.005726	-5.261792	56.6129	43.3871
PRB1	5542	0.391	0.566667	-0.81797	0.026068	-5.399308	56.66667	43.33333
RIT2	6014	0.378	0.566717	-0.81773	0.01175	-8.878674	56.67166	43.32834
GALR3	8484	0.39	0.56686	-0.81704	0.005778	-9.591326	56.68605	43.31395
SLC2A2	6514	0.457	0.568408	-0.80957	0.004402	-7.44183	56.8408	43.1592
POLR2D	5433	0.343	0.568823	-0.80757	0.010047	-26.59997	56.88226	43.11774
SCGF	6320	0.486	0.569087	-0.80629	0.002408	-5.034898	56.90867	43.09133
TUBB4	10381	0.362	0.569182	-0.80583	0.010586	-14.39895	56.91824	43.08176
FOX E1	2304	0.433	0.569737	-0.80315	0.000011	-12.66197	56.97368	43.02632
ANXA11	311	0.379	0.569925	-0.80225	0.000874	-20.36203	56.99248	43.00752
NXT1	29107	0.459	0.570896	-0.79756	0.001913	-8.640025	57.08955	42.91045
NTF5	4909	0.488	0.571429	-0.79499	0.000457	-7.154722	57.14286	42.85714

BID	637	0.425	0.572005	-0.79221	0.012415	-17.17511	57.20054	42.79946
FBXO22	26263	0.43	0.573333	-0.7858	0.019878	-5.380238	57.33333	42.66667
PIP3AP	54545	0.396	0.573913	-0.783	0.032991	-44.44497	57.3913	42.6087
TNFSF7	970	0.384	0.57485	-0.77848	0.00857	-11.02488	57.48503	42.51497
IFNA4	3441	0.398	0.575145	-0.77706	0.00375	-9.738373	57.51445	42.48555
GPR114	221188	0.396	0.575581	-0.77495	0.007451	-8.723521	57.55814	42.44186
HSPB1	3315	0.349	0.575908	-0.77337	0.001289	-5.361356	57.59076	42.40924
SFRS11	9295	0.365	0.577532	-0.76554	0.001324	-6.488545	57.75316	42.24684
TMF1	7110	0.368	0.578616	-0.7603	0.0154	-6.575857	57.86164	42.13836
PCSK1	5122	0.349	0.578773	-0.75955	0.024136	-9.052594	57.87728	42.12272
RBM15	64783	0.329	0.579225	-0.75736	0.008247	-23.78825	57.92254	42.07746
FBXO6	26270	0.435	0.58	-0.75362	0.003128	-5.151044	58	42
UCP2	7351	0.44	0.580475	-0.75133	0.010884	-5.146285	58.04749	41.95251
TECTA	7007	0.429	0.580514	-0.75114	0.003173	-8.575755	58.05142	41.94858
SP100	6672	0.367	0.580696	-0.75027	0.004624	-5.276937	58.06962	41.93038
RFX5	5993	0.295	0.580709	-0.7502	0.014538	-7.851201	58.07087	41.92913
PTPRN	5798	0.364	0.58147	-0.74653	0.004141	-7.754066	58.14696	41.85304
CASP4	837	0.332	0.582456	-0.74177	0.00099	-10.01382	58.24561	41.75439
PRDM11	56981	0.402	0.582609	-0.74104	0.025481	-12.95922	58.26087	41.73913
SFRP5	6425	0.413	0.583333	-0.73754	0.007256	-13.64226	58.33333	41.66667
KIF2	3796	0.373	0.583725	-0.73565	0.001856	-13.78945	58.37246	41.62754
TIRAP	114609	0.395	0.583888	-0.73486	0.006393	-6.583535	58.38877	41.61123
C20ORF18	10616	0.438	0.584	-0.73432	0.003765	-11.37537	58.4	41.6
CCT4	10575	0.479	0.584146	-0.73361	0.007808	-7.211695	58.41463	41.58537
WNT1	7471	0.396	0.585366	-0.72773	0.014861	-5.007421	58.53659	41.46341
SOX3	6658	0.393	0.585693	-0.72615	0.013869	-5.938394	58.5693	41.4307
KCNAB2	8514	0.4	0.58651	-0.72221	0.000925	-6.776242	58.65103	41.34897
ANAPC11	51529	0.44	0.586667	-0.72145	0.008285	-38.24449	58.66667	41.33333
ITGAE	3682	0.422	0.586926	-0.7202	0.024673	-9.317421	58.69263	41.30737
GRP58	2923	0.344	0.587031	-0.71969	0.000979	-10.13337	58.70307	41.29693
SMPD1	6609	0.327	0.588129	-0.71439	0.004165	-11.24751	58.81295	41.18705
BLMH	642	0.437	0.588156	-0.71426	0.02528	-5.146869	58.81561	41.18439
GUCA1C	9626	0.448	0.589474	-0.7079	0.002923	-7.46647	58.94737	41.05263
TAF5	6877	0.375	0.589623	-0.70719	0.002891	-6.654301	58.96226	41.03774
ASAH1	427	0.295	0.59	-0.70536	0.008247	-8.196132	59	41
M6PR	4074	0.418	0.590395	-0.70346	0.004476	-12.56115	59.03955	40.96045
SCAMP2	10066	0.355	0.590682	-0.70207	0.023823	-8.989092	59.06822	40.93178
MMP8	4317	0.371	0.590764	-0.70168	0.001494	-5.14239	59.07643	40.92357
ABCC6	368	0.475	0.590796	-0.70152	0.000697	-7.027035	59.0796	40.9204
TBX18	9096	0.337	0.591228	-0.69944	0.002326	-7.185891	59.12281	40.87719
GABRB1	2560	0.395	0.591317	-0.69901	0.005187	-6.59281	59.13174	40.86826
PIP5K3	200576	0.357	0.59204	-0.69552	0.014542	-44.81432	59.20398	40.79602
HTATIP	10524	0.402	0.592047	-0.69549	0.017253	-19.36841	59.20471	40.79529
KCNK13	56659	0.404	0.592375	-0.6939	0.006037	-5.482393	59.23754	40.76246
IL5	3567	0.426	0.59249	-0.69335	0.021088	-11.66467	59.24896	40.75104
HOXA11	3207	0.323	0.592661	-0.69253	0.053048	-5.352768	59.26606	40.73395

PTPN2	5771	0.377	0.592767	-0.69201	0.015403	-10.44103	59.27673	40.72327
TPTE2	93492	0.379	0.593114	-0.69034	0.001353	-30.38515	59.31142	40.68858
RING1	6015	0.445	0.593333	-0.68928	0.003995	-5.101713	59.33333	40.66667
GSTT2	2953	0.451	0.593421	-0.68885	0.00228	-5.488301	59.34211	40.65789
USP14	9097	0.381	0.593458	-0.68868	0.027159	-7.766395	59.34579	40.65421
SUOX	6821	0.396	0.593703	-0.68749	0.003041	-11.4732	59.37031	40.62969
GATM	2628	0.348	0.593857	-0.68675	0.008536	-11.10947	59.38567	40.61433
GNRH2	2797	0.411	0.593931	-0.6864	0.004711	-14.94282	59.39306	40.60694
GAL3ST1	9514	0.319	0.594041	-0.68586	8.62E-05	-7.5949	59.4041	40.5959
CPA2	1358	0.319	0.594041	-0.68586	0.007241	-6.678488	59.4041	40.5959
CAMKIINA	55450	0.32	0.595903	-0.67688	0.000763	-16.33686	59.59032	40.40968
LPHA								
CYP7B1	9420	0.32	0.595903	-0.67688	0.00206	-11.08904	59.59032	40.40968
GPX2	2877	0.405	0.596465	-0.67416	0.022959	-6.712635	59.64654	40.35346
CHM	1121	0.37	0.596774	-0.67267	0.000703	-6.340487	59.67742	40.32258
ADM	133	0.404	0.597191	-0.67066	0.00256	-12.0591	59.71914	40.28086
SNRPN	6638	0.378	0.598101	-0.66627	0.001967	-6.072173	59.81013	40.18987
PKD2	5311	0.462	0.598446	-0.66461	0.001118	-8.225629	59.84456	40.15544
TNFRSF19L	84957	0.405	0.59867	-0.66353	0.004352	-17.67579	59.86696	40.13304
CEACAM7	1087	0.453	0.599206	-0.66094	0.001828	-6.827469	59.92063	40.07937
ICSBP1	3394	0.388	0.599228	-0.66083	0.002205	-32.18248	59.92278	40.07722
USP35	57558	0.385	0.599688	-0.65861	0.003717	-5.021847	59.96885	40.03115
PGGT1B	5229	0.362	0.600332	-0.6555	0.01741	-33.1319	60.03317	39.96683
TREX2	11219	0.382	0.600629	-0.65407	0.010905	-7.064794	60.06289	39.93711
CHI3L2	1117	0.323	0.60149	-0.64992	0.003702	-10.15749	60.14898	39.85102
SCAMP3	10067	0.484	0.60199	-0.6475	0.00891	-5.261594	60.19901	39.801
TAF7	6879	0.383	0.602201	-0.64648	0.00311	-7.561227	60.22013	39.77987
SAH	6296	0.335	0.602518	-0.64495	0.00269	-5.628069	60.2518	39.7482
COL1A2	1278	0.455	0.602649	-0.64432	0.004665	-9.98344	60.2649	39.7351
WHSC1L1	54904	0.452	0.602667	-0.64424	0.004881	-13.23212	60.26667	39.73333
PRPF4	9128	0.343	0.603873	-0.63841	0.008733	-8.262118	60.38732	39.61268
HNF4A	3172	0.366	0.60396	-0.63799	0.020356	-6.463759	60.39604	39.60396
CENPE	1062	0.386	0.604069	-0.63747	0.00013	-7.361583	60.40689	39.59311
PCDH11X	27328	0.463	0.604439	-0.63568	0.002162	-6.686163	60.44386	39.55614
IL1RN	3557	0.435	0.605007	-0.63294	0.019627	-5.172676	60.5007	39.4993
PDCD6	10016	0.465	0.60705	-0.62308	0.003179	-7.949269	60.70496	39.29504
RNPS1	10921	0.345	0.607394	-0.62142	0.014075	-8.788432	60.73944	39.26056
HAT1	8520	0.462	0.607895	-0.619	0.0027	-9.598854	60.78947	39.21053
ALDR16	55586	0.304	0.608	-0.6185	0.008907	-7.375571	60.8	39.2
MT2A	4502	0.466	0.608355	-0.61678	0.002375	-6.041	60.83551	39.16449
CEL	1056	0.46	0.608466	-0.61625	0.007611	-18.95158	60.84656	39.15344
DECR1	1666	0.327	0.608939	-0.61397	0.000272	-9.319246	60.89385	39.10615
USP21	27005	0.391	0.609034	-0.61351	0.005594	-5.68661	60.90343	39.09657
APLP2	334	0.344	0.609929	-0.60919	0.016447	-5.266719	60.99291	39.00709
TBP	6908	0.388	0.610063	-0.60854	0.002073	-5.262317	61.00629	38.99371
PRKCDBP	112464	0.421	0.610145	-0.60815	0.044653	-15.35354	61.01449	38.98551
CDC23	8697	0.328	0.610801	-0.60498	0.000452	-11.78846	61.08007	38.91993

SLIT1	6585	0.41	0.611028	-0.60388	0.00751	-10.28615	61.10283	38.89717
MMP2	4313	0.384	0.611465	-0.60178	0.003405	-8.096917	61.1465	38.8535
ELYS	25909	0.466	0.611549	-0.60137	0.004982	-5.790103	61.15486	38.84514
PRG4	10216	0.422	0.611594	-0.60115	0.030697	-11.36187	61.15942	38.84058
SCNN1G	6340	0.492	0.61194	-0.59948	0.001873	-5.444869	61.19403	38.80597
FBXL3P	26223	0.459	0.612	-0.59919	0.020987	-5.066761	61.2	38.8
DNMT3B	1789	0.467	0.612861	-0.59504	0.01247	-30.49265	61.28609	38.71391
FRS2	10818	0.38	0.612903	-0.59483	0.00394	-7.429292	61.29032	38.70968
TUBD1	51174	0.39	0.613208	-0.59337	0.001156	-5.980675	61.32075	38.67925
SDSL	113675	0.341	0.613309	-0.59287	0.006016	-8.661845	61.33094	38.66906
TP53INP1	94241	0.382	0.614148	-0.58883	0.023966	-15.29581	61.41479	38.58521
SLC12A6	9990	0.494	0.614428	-0.58748	0.007284	-5.720918	61.44279	38.55721
PLEK	5341	0.424	0.614493	-0.58716	0.028666	-8.791199	61.44928	38.55072
CBLC	23624	0.461	0.614667	-0.58632	0.011983	-17.13554	61.46667	38.53333
AKAP28	158798	0.477	0.614691	-0.58621	0.006247	-7.341295	61.46907	38.53093
KIF3A	11127	0.393	0.615023	-0.5846	0.003697	-12.688	61.50235	38.49765
FBXO2	26232	0.462	0.616	-0.57989	0.012994	-14.3529	61.6	38.4
POLR2B	5431	0.371	0.616279	-0.57854	0.014596	-5.210678	61.62791	38.37209
PORCN	64840	0.371	0.616279	-0.57854	0.007202	-5.191093	61.62791	38.37209
ABCB4	5244	0.41	0.616541	-0.57728	0.009128	-5.143859	61.65414	38.34586
CACNA1C	775	0.476	0.61658	-0.57709	0.005557	-14.39598	61.65803	38.34197
TFRC	7037	0.343	0.616906	-0.57551	0.004559	-8.48325	61.69065	38.30935
GSTA1	2938	0.362	0.617747	-0.57146	0.008595	-7.526805	61.77474	38.22526
DERP6	23587	0.508	0.618005	-0.57021	0.001415	-7.537025	61.80049	38.19951
PR48	28227	0.42	0.618557	-0.56755	0.017372	-19.70832	61.85567	38.14433
DRD2	1813	0.426	0.619186	-0.56451	0.002169	-21.25937	61.9186	38.0814
CST3	1471	0.509	0.619221	-0.56434	0.00548	-10.83556	61.92214	38.07786
TIMP4	7079	0.384	0.619355	-0.5637	0.002248	-11.53687	61.93548	38.06452
EXO1	9156	0.472	0.619423	-0.56337	0.004744	-5.469204	61.94226	38.05774
GIP	2695	0.429	0.619942	-0.56086	0.006063	-7.808672	61.99422	38.00578
KCNT1	57582	0.423	0.620235	-0.55945	0.00513	-8.731372	62.02346	37.97654
CDC20	991	0.469	0.62037	-0.5588	0.023813	-5.225386	62.03704	37.96296
DAAM1	23002	0.51	0.620438	-0.55847	0.005464	-8.133721	62.0438	37.9562
ADAMTS6	11174	0.375	0.620861	-0.55643	0.009059	-28.62396	62.08609	37.91391
LOC25303 9	253039	0.452	0.620879	-0.55634	0.001046	-6.211952	62.08791	37.91209
RAB38	23682	0.422	0.621502	-0.55334	0.015167	-5.61574	62.15022	37.84978
PBX1	5087	0.422	0.621502	-0.55334	0.029795	-5.346945	62.15022	37.84978
WWP1	11059	0.428	0.622093	-0.55048	0.013577	-5.190325	62.2093	37.7907
TAF1B	9014	0.396	0.622642	-0.54784	0.001869	-5.598169	62.26415	37.73585
AUH	549	0.355	0.622807	-0.54704	0.002804	-6.125695	62.2807	37.7193
MSR1	4481	0.441	0.622881	-0.54668	0.009621	-27.89753	62.28814	37.71186
RAB13	5872	0.423	0.622975	-0.54623	0.014555	-24.95034	62.2975	37.7025
PTPRZ1	5803	0.39	0.623003	-0.54609	0.004626	-10.8064	62.30032	37.69968
GART	2618	0.366	0.624573	-0.53851	0.014067	-5.989987	62.45734	37.54266
P11	8909	0.377	0.625207	-0.53545	0.014875	-7.270893	62.52073	37.47927
APBB1	322	0.416	0.625564	-0.53373	0.008753	-6.078951	62.55639	37.44361

HFE	3077	0.358	0.625874	-0.53224	0.012442	-104.2352	62.58741	37.41259
WBSCR14	51085	0.353	0.625887	-0.53218	0.030366	-7.772109	62.58865	37.41135
FECH	2235	0.477	0.625984	-0.53171	0.010271	-12.50759	62.59843	37.40157
S100A2	6273	0.356	0.626761	-0.52796	0.006626	-5.494118	62.67606	37.32394
CR1	1378	0.469	0.627005	-0.52678	0.016404	-5.026625	62.70053	37.29947
IFNA21	3452	0.434	0.627168	-0.52599	0.004409	-5.08789	62.71676	37.28324
DHX16	8449	0.478	0.627297	-0.52537	0.008523	-5.350296	62.72966	37.27034
GADD45A	1647	0.464	0.627876	-0.52258	0.013945	-11.03942	62.78755	37.21245
TCL1A	8115	0.358	0.62807	-0.52164	0.002507	-6.787897	62.80702	37.19298
G6PC2	57818	0.407	0.628086	-0.52156	0.027621	-6.818721	62.80864	37.19136
BBS1	582	0.467	0.628533	-0.5194	0.006908	-5.647013	62.8533	37.1467
IMP-3	10643	0.408	0.62963	-0.51411	0.028966	-8.266795	62.96296	37.03704
GUSB	2990	0.369	0.629693	-0.51381	0.000513	-6.699502	62.96928	37.03072
37865	55964	0.398	0.629747	-0.51355	0.003304	-9.180766	62.97468	37.02532
GLTSCR1	29998	0.479	0.630263	-0.51106	0.003186	-6.928768	63.02632	36.97368
LOC90557	90557	0.459	0.630495	-0.50994	0.001385	-6.344777	63.04945	36.95055
TBX2	6909	0.401	0.630503	-0.5099	0.012462	-17.98354	63.05031	36.94969
MGST2	4258	0.391	0.630645	-0.50921	0.00487	-5.615612	63.06452	36.93548
GPD1	2819	0.403	0.630673	-0.50908	0.001084	-9.685146	63.06729	36.93271
TXNDC4	23071	0.429	0.631811	-0.50358	0.032553	-5.34341	63.18115	36.81885
DNCH1	1778	0.392	0.632258	-0.50143	0.003074	-6.703714	63.22581	36.77419
IAPP	3375	0.438	0.632948	-0.4981	0.004051	-9.047828	63.2948	36.7052
CD38	952	0.34	0.633147	-0.49714	0.0061	-12.94255	63.31471	36.68529
DLL3	10683	0.439	0.634393	-0.49112	0.00573	-7.017202	63.43931	36.56069
SPG4	6683	0.401	0.634494	-0.49064	0.000508	-7.389258	63.44937	36.55063
CIDEB	27141	0.41	0.634675	-0.48976	0.009413	-6.525546	63.46749	36.53251
HTATIP2	10553	0.411	0.634749	-0.48941	0.001624	-7.915751	63.4749	36.5251
HIF1AN	55662	0.372	0.634812	-0.4891	0.004009	-22.29754	63.48123	36.51877
SPRY1	10252	0.426	0.634873	-0.48881	0.027349	-5.012149	63.48733	36.51267
NEIL1	79661	0.402	0.635071	-0.48785	0.00478	-5.158233	63.50711	36.49289
FARSLA	2193	0.517	0.635135	-0.48754	0.000871	-5.253949	63.51351	36.48649
GDF15	9518	0.44	0.635838	-0.48415	0.015364	-5.540358	63.58382	36.41619
HRG	3273	0.4	0.63593	-0.48371	0.036744	-5.959632	63.593	36.407
KCNA6	3742	0.491	0.63601	-0.48332	0.00555	-8.023979	63.60104	36.39896
SDHB	6390	0.432	0.63623	-0.48226	0.010893	-10.64339	63.62298	36.37703
JAG1	182	0.481	0.637086	-0.47813	0.006147	-7.595326	63.70861	36.29139
ABCC5	10057	0.483	0.637203	-0.47756	0.011729	-37.89874	63.72032	36.27968
KCNB2	9312	0.492	0.637306	-0.47707	0.001559	-9.305108	63.73057	36.26943
PPP1CB	5500	0.399	0.63738	-0.47671	0.01589	-9.394198	63.73802	36.26198
CENPJ	55835	0.482	0.637566	-0.47581	0.021973	-6.230325	63.75661	36.24339
SFRP1	6422	0.452	0.638418	-0.4717	0.015987	-8.329509	63.84181	36.15819
PPP2CZ	333926	0.408	0.638498	-0.47132	0.006363	-8.054434	63.84977	36.15023
PIWIL2	55124	0.441	0.63913	-0.46826	0.059427	-5.512136	63.91304	36.08696
GTF3C4	9329	0.434	0.639175	-0.46805	0.02521	-7.692891	63.91753	36.08247
SLC9A3R2	9351	0.404	0.639241	-0.46773	0.004313	-5.424358	63.92405	36.07595
SP4	6671	0.325	0.639764	-0.46521	0.012985	-11.85675	63.97638	36.02362

SRY	6736	0.407	0.639937	-0.46437	0.004494	-6.059079	63.99371	36.00629
PTPN14	5784	0.409	0.640063	-0.46376	0.000415	-5.316182	64.00626	35.99374
OGDH	4967	0.386	0.640133	-0.46342	0.01087	-10.20243	64.01327	35.98673
GATA2	2624	0.349	0.640367	-0.46229	0.041434	-5.783533	64.0367	35.9633
CGI-63	51102	0.366	0.642105	-0.45391	0.003376	-5.825059	64.21053	35.78947
THBS3	7059	0.366	0.642105	-0.45391	0.003102	-5.774393	64.21053	35.78947
SFTPC	6440	0.431	0.642325	-0.45285	0.006814	-7.049629	64.23249	35.76751
CART	9607	0.527	0.642683	-0.45112	0.005776	-5.123893	64.26829	35.73171
TNFRSF17	608	0.436	0.644494	-0.44238	0.009327	-9.744529	64.44937	35.55063
TBX1	6899	0.368	0.645614	-0.43697	0.000955	-10.66218	64.5614	35.4386
UBASH3A	53347	0.359	0.645683	-0.43664	0.002445	-5.641896	64.56835	35.43165
MS4A7	58475	0.458	0.646893	-0.4308	0.011604	-43.64379	64.68927	35.31073
RBMY1A1	5940	0.389	0.647255	-0.42905	0.027974	-5.874864	64.72546	35.27454
TRPV4	59341	0.521	0.64801	-0.42541	0.002592	-5.495918	64.801	35.19901
KRAS2	3845	0.407	0.648089	-0.42503	0.006123	-5.643693	64.80892	35.19108
FBXW7	55294	0.487	0.649333	-0.41902	0.021107	-6.71655	64.93333	35.06667
TUBG1	7283	0.413	0.649371	-0.41884	0.00751	-19.39338	64.93711	35.06289
ITGA10	8515	0.467	0.649513	-0.41815	0.028366	-5.563248	64.95132	35.04868
PTPN12	5782	0.407	0.65016	-0.41503	0.008657	-8.22118	65.01597	34.98403
PPP1R9B	84687	0.409	0.650238	-0.41465	0.018651	-5.102242	65.02385	34.97615
SCML1	6322	0.331	0.651575	-0.40821	0.013156	-7.940338	65.15748	34.84252
ANGPTL3	27329	0.404	0.651613	-0.40802	0.004012	-7.614587	65.16129	34.83871
BUB3	9184	0.485	0.652759	-0.40249	0.005191	-5.638968	65.27591	34.72409
RRAS2	22800	0.436	0.653673	-0.39808	0.008258	-6.152286	65.36732	34.63268
HCRT1	3061	0.45	0.65407	-0.39616	0.012766	-6.391438	65.40698	34.59302
ILT10	79166	0.424	0.654321	-0.39495	0.02119	-5.138966	65.4321	34.5679
HOXD9	3235	0.424	0.654826	-0.39251	0.001656	-12.30215	65.48263	34.51737
CNTNAP3	79937	0.484	0.654939	-0.39197	0.017356	-10.5894	65.49391	34.50609
FGF2	2247	0.509	0.655084	-0.39127	0.005755	-11.16376	65.50837	34.49163
BCAS1	8537	0.487	0.655451	-0.3895	0.016533	-5.650917	65.54509	34.45491
RFX1	5989	0.333	0.655512	-0.38921	0.022045	-5.904292	65.55118	34.44882
ACTN2	88	0.485	0.656292	-0.38544	0.009179	-5.900483	65.62923	34.37077
MAD1L1	8379	0.478	0.656593	-0.38399	0.00161	-18.74229	65.65934	34.34066
LAMB1	3912	0.501	0.658344	-0.37554	0.003206	-6.382704	65.83443	34.16557
MGC4083	84617	0.419	0.658805	-0.37331	0.015587	-6.535014	65.8805	34.1195
CNOT3	4849	0.467	0.659605	-0.36945	0.05783	-5.490183	65.96045	34.03955
FLJ11011	55284	0.448	0.659794	-0.36854	0.037019	-5.161739	65.97938	34.02062
DD5	51366	0.495	0.66	-0.36755	0.004209	-6.693082	66	34
CDC16	8881	0.499	0.660053	-0.36729	0.013009	-9.191924	66.00529	33.99471
TREM1	54210	0.447	0.660754	-0.36391	0.006355	-47.39637	66.07539	33.92461
TNFRSF10D	8793	0.448	0.662232	-0.35677	0.001549	-6.317431	66.22321	33.77679
ADH1A	124	0.4	0.662252	-0.35668	0.011996	-29.08348	66.22517	33.77483
RANBP2	5903	0.442	0.662669	-0.35467	0.001924	-6.233819	66.26687	33.73313
AIPL1	23746	0.411	0.662903	-0.35353	0.006074	-5.788246	66.29032	33.70968
MDS1	4197	0.43	0.664093	-0.34779	3.71E-05	-6.434963	66.40927	33.59073
NMB	4828	0.459	0.664255	-0.34701	0.002858	-6.222817	66.42547	33.57453

CSF3R	1441	0.497	0.664439	-0.34612	0.028193	-5.08804	66.44385	33.55615
LTBP3	4054	0.484	0.664835	-0.34421	7.42E-06	-7.459275	66.48352	33.51648
DUT	1854	0.507	0.665354	-0.34171	0.032188	-7.363178	66.53543	33.46457
SYNCRIP	10492	0.421	0.666139	-0.33792	0.000226	-7.989275	66.61392	33.38608
CASP6	839	0.359	0.668529	-0.32638	0.008751	-6.611197	66.85289	33.14711
HMGCL	3155	0.392	0.668942	-0.32439	0.017254	-5.079963	66.8942	33.1058
SHBG	6462	0.423	0.669304	-0.32265	0.002499	-15.61022	66.93038	33.06962
PRDX4	10549	0.455	0.670103	-0.31879	0.023226	-25.92459	67.01031	32.98969
COL15A1	1306	0.551	0.670316	-0.31776	0.010325	-5.118602	67.03163	32.96837
GNRH1	2796	0.464	0.67052	-0.31677	0.020467	-10.70272	67.05202	32.94798
ANXA3	306	0.456	0.671576	-0.31168	0.010496	-7.957947	67.15758	32.84242
EZH2	2146	0.547	0.67199	-0.30968	0.006584	-5.062724	67.19902	32.80098
BAAT	570	0.336	0.672	-0.30963	0.006067	-23.97806	67.2	32.8
FBXL6	26233	0.504	0.672	-0.30963	0.016679	-14.19435	67.2	32.8
SET7	80854	0.374	0.672662	-0.30644	0.01087	-6.417657	67.26619	32.73381
IL6R	3570	0.484	0.673157	-0.30405	0.036736	-8.518548	67.31572	32.68428
ADAM21	8747	0.407	0.673841	-0.30075	0.021068	-8.245035	67.38411	32.61589
UGT1A9	54600	0.407	0.673841	-0.30075	0.010819	-6.66059	67.38411	32.61589
RASGRF1	5923	0.437	0.674383	-0.29813	0.073731	-7.832483	67.43827	32.56173
ATP7B	540	0.429	0.674528	-0.29743	0.021527	-10.09779	67.45283	32.54717
HCN1	348980	0.521	0.67487	-0.29578	0.0002	-5.552584	67.48705	32.51295
SOX18	54345	0.344	0.677165	-0.28471	0.015235	-19.13845	67.71654	32.28346
SCA1	6310	0.428	0.677215	-0.28446	0.006099	-6.768423	67.72152	32.27848
GRIPAP1	56850	0.515	0.677632	-0.28246	0.005407	-5.551027	67.76316	32.23684
MTMR2	8898	0.425	0.678914	-0.27627	0.004347	-5.79187	67.89137	32.10863
PTPRK	5796	0.425	0.678914	-0.27627	0.00875	-5.327754	67.89137	32.10863
DUSP15	128853	0.434	0.679186	-0.27495	0.000988	-9.473817	67.91862	32.08138
ACADS	35	0.34	0.68	-0.27103	0.00671	-17.38047	68	32
MCP	4179	0.482	0.680791	-0.26721	0.025833	-5.138958	68.0791	31.9209
CHD2	1106	0.389	0.682456	-0.25917	0.001062	-11.78587	68.24561	31.75439
HINT1	3094	0.519	0.682895	-0.25706	0.00301	-5.340982	68.28947	31.71053
GLTSCR2	29997	0.519	0.682895	-0.25706	0.00654	-5.065641	68.28947	31.71053
FBXO11	80204	0.513	0.684	-0.25172	0.022428	-8.428699	68.4	31.6
PTP4A2	8073	0.429	0.685304	-0.24543	0.021189	-7.149044	68.53035	31.46965
CCNC	892	0.562	0.685366	-0.24513	0.005313	-10.99822	68.53659	31.46341
UGT2B4	7363	0.414	0.68543	-0.24482	0.00468	-5.218795	68.54305	31.45695
MECP2	4204	0.445	0.687259	-0.23599	0.002359	-145.6465	68.72587	31.27413
SSR2	6746	0.473	0.6875	-0.23483	0.012696	-7.516618	68.75	31.25
PPP1R14A	94274	0.414	0.687708	-0.23383	0.026625	-56.82552	68.77076	31.22924
CCNT1	904	0.564	0.687805	-0.23336	0.004667	-6.378358	68.78049	31.21951
SLC1A1	6505	0.553	0.687811	-0.23333	0.003508	-12.61576	68.78109	31.21891
AKR7A2	8574	0.344	0.688	-0.23242	0.00018	-5.218511	68.8	31.2
FBXL9	26231	0.517	0.689333	-0.22598	0.011897	-86.55073	68.93333	31.06667
TPO	7173	0.469	0.690722	-0.21928	0.02164	-8.750394	69.07216	30.92784
ACOX2	8309	0.524	0.691293	-0.21653	0.025787	-5.605973	69.12929	30.87071
CCK	885	0.518	0.692513	-0.21064	0.011494	-5.611104	69.25134	30.74866

SPAM1	6677	0.462	0.692654	-0.20996	0.002235	-6.606821	69.26537	30.73463
RNF6	6049	0.477	0.693314	-0.20677	0.015499	-5.746724	69.3314	30.6686
RAB11B	9230	0.471	0.693667	-0.20507	0.019019	-24.14731	69.36672	30.63328
CTSF	8722	0.373	0.6946	-0.20057	0.000708	-6.325285	69.45996	30.54004
FBXL4	26235	0.521	0.694667	-0.20024	0.030524	-5.0333	69.46667	30.53333
PHTF1	10745	0.353	0.694882	-0.19921	0.005649	-5.945803	69.48819	30.51181
TCEB3	6924	0.442	0.694969	-0.19879	0.009001	-6.88086	69.49686	30.50314
ZNFN1A1	10320	0.392	0.695035	-0.19846	0.023338	-7.025409	69.50355	30.49645
APPBP2	10513	0.431	0.695161	-0.19786	0.003782	-7.889735	69.51613	30.48387
SLC4A1	6521	0.559	0.695274	-0.19731	0.008323	-8.265239	69.52736	30.47264
GABRA4	2557	0.465	0.696108	-0.19329	0.0113	-5.857335	69.61078	30.38922
HERC6	55008	0.447	0.696262	-0.19255	0.025035	-14.84399	69.62617	30.37383
NQO3A2	51706	0.374	0.696462	-0.19158	0.010475	-8.588056	69.64618	30.35382
DHFR	1719	0.404	0.696552	-0.19115	0.002133	-5.809308	69.65517	30.34483
TEBP	10728	0.465	0.697151	-0.18825	0.006142	-14.42541	69.71514	30.28486
BTRC	8945	0.523	0.697333	-0.18737	0.00854	-13.23035	69.73333	30.26667
IFIT5	24138	0.423	0.69802	-0.18406	0.020974	-6.889052	69.80198	30.19802
FBXL3A	26224	0.524	0.698667	-0.18094	0.021795	-10.43539	69.86667	30.13333
ACY1	95	0.35	0.7	-0.17451	0.009459	-6.526358	70	30
CTSO	1519	0.376	0.700186	-0.17361	0.0103	-8.948766	70.01862	29.98138
DMC1	11144	0.534	0.700787	-0.17071	0.036903	-7.593531	70.07874	29.92126
PLAG1	5324	0.356	0.700787	-0.17071	0.025765	-5.827037	70.07874	29.92126
POU4F1	5457	0.356	0.700787	-0.17071	0.018828	-5.510901	70.07874	29.92126
GLI3	2737	0.382	0.700917	-0.17008	0.067609	-5.818863	70.09174	29.90826
FBXL5	26234	0.526	0.701333	-0.16807	0.017817	-7.215688	70.13333	29.86667
SURB7	9412	0.435	0.701613	-0.16672	0.003775	-12.56755	70.16129	29.83871
CD58	965	0.455	0.704334	-0.15359	0.006924	-9.532035	70.43344	29.56656
RXRG	6258	0.602	0.704918	-0.15077	0.007918	-14.95133	70.4918	29.5082
RNUT1	10073	0.401	0.705986	-0.14562	0.012002	-5.707223	70.59859	29.40141
PTPRE	5791	0.442	0.70607	-0.14521	0.029516	-6.272461	70.60703	29.39297
SH3GL2	6456	0.471	0.706147	-0.14484	0.012629	-5.938033	70.61469	29.38531
LTBP4	8425	0.489	0.70767	-0.13749	0.017747	-5.10567	70.767	29.233
IFNA2	3440	0.49	0.708092	-0.13545	0.025036	-5.19809	70.80925	29.19075
BCL2	596	0.471	0.708271	-0.13459	0.003275	-5.144365	70.82707	29.17293
MPO	4353	0.481	0.708395	-0.13399	0.03104	-6.608856	70.83947	29.16053
ADAMTS9	56999	0.428	0.708609	-0.13296	0.007135	-6.645811	70.86093	29.13907
LOR	4014	0.524	0.709066	-0.13075	0.0153	-21.66488	70.90663	29.09337
SNURF	8926	0.476	0.709389	-0.12919	0.013337	-9.202536	70.9389	29.0611
VGf	7425	0.474	0.709581	-0.12827	0.01679	-20.84704	70.95808	29.04192
HNF4G	3174	0.582	0.710623	-0.12324	0.022716	-5.339651	71.06227	28.93773
PTPRD	5789	0.445	0.710863	-0.12208	0.020247	-9.276097	71.08626	28.91374
PVRL2	5819	0.504	0.711864	-0.11725	0.010251	-12.75873	71.18644	28.81356
RAMP3	10268	0.504	0.711864	-0.11725	0.004017	-5.443975	71.18644	28.81356
SNAI1	6615	0.45	0.712025	-0.11647	0.007998	-6.031885	71.20253	28.79747
PPARG	5468	0.609	0.713115	-0.11121	0.007879	-6.048307	71.31148	28.68852
GNMT	27232	0.542	0.713158	-0.11101	0.003269	-9.202014	71.31579	28.68421

APG7L	10533	0.418	0.713311	-0.11027	0.005103	-15.39838	71.33106	28.66894
CBR1	873	0.585	0.713415	-0.10977	0.014963	-5.363934	71.34146	28.65854
PPP2R5D	5528	0.447	0.714058	-0.10666	0.02387	-6.965958	71.40575	28.59425
ZNF198	7750	0.403	0.714539	-0.10434	0.02459	-5.126948	71.4539	28.5461
WNT2B	7482	0.484	0.715447	-0.09996	0.006907	-76.23037	71.54472	28.45528
TNFRSF1B	7133	0.611	0.715457	-0.09991	0.008579	-17.20778	71.54567	28.45433
PDCD8	9131	0.432	0.716418	-0.09527	0.050341	-7.925273	71.64179	28.35821
AREG	374	0.485	0.716925	-0.09282	0.008759	-24.23146	71.69254	28.30746
PDLIM4	8572	0.408	0.71831	-0.08614	0.020911	-9.508596	71.83099	28.16901
CHRNA4	1137	0.48	0.718563	-0.08492	0.004557	-5.162771	71.85629	28.14371
FBXL2	25827	0.539	0.718667	-0.08442	0.009014	-10.37233	71.86667	28.13333
TNFSF4	7292	0.481	0.72006	-0.0777	0.017307	-8.457503	72.00599	27.99401
CNTN1	1272	0.466	0.721362	-0.07141	0.023048	-5.262656	72.13622	27.86378
SORCS2	57537	0.591	0.721612	-0.07021	0.028086	-6.07745	72.16117	27.83883
FEN1	2237	0.55	0.721785	-0.06937	0.02299	-8.392404	72.17848	27.82152
HRMT1L1	3275	0.424	0.723549	-0.06086	0.007618	-55.37495	72.35495	27.64505
PEO1	56652	0.437	0.72471	-0.05526	0.024264	-14.36836	72.47098	27.52902
ANG	283	0.438	0.725166	-0.05306	0.034316	-5.11676	72.51656	27.48344
GGPS1	9453	0.425	0.725256	-0.05262	0.018092	-5.287058	72.5256	27.4744
GSTM3	2947	0.426	0.726962	-0.04438	0.019235	-6.994391	72.69625	27.30375
RHBDL6	79651	0.566	0.728443	-0.03724	0.027572	-8.263845	72.84427	27.15573
CHD3	1107	0.468	0.728972	-0.03469	0.015404	-5.488182	72.8972	27.1028
DCT	1638	0.556	0.729659	-0.03137	0.020662	-8.636441	72.96588	27.03412
CYP1A2	1544	0.6	0.729927	-0.03008	0.008927	-10.29924	72.9927	27.0073
HEXB	3074	0.428	0.730375	-0.02791	0.010947	-30.96374	73.03754	26.96246
PPAT	5471	0.44	0.730897	-0.0254	0.0308	-17.99192	73.0897	26.9103
PDE2A	5138	0.441	0.731343	-0.02324	0.044989	-11.76174	73.13433	26.86567
FAM64A	54478	0.556	0.731579	-0.02211	0.007748	-5.377316	73.15789	26.84211
PLXNB3	5365	0.518	0.731638	-0.02182	0.010106	-10.73398	73.16384	26.83616
BLVRA	644	0.393	0.731844	-0.02083	0.005636	-9.655716	73.18436	26.81564
AHCYL1	10768	0.366	0.732	-0.02007	0.010501	-12.5198	73.2	26.8
MYO6	4646	0.455	0.733871	-0.01104	0.002156	-10.89551	73.3871	26.6129
SIX2	10736	0.373	0.734252	-0.00921	0.024706	-8.280039	73.4252	26.5748
GOLGB1	2804	0.559	0.735526	-0.00306	0.004448	-5.663839	73.55263	26.44737
SALL1	6299	0.418	0.735915	-0.00118	0.015502	-8.102399	73.59155	26.40845
CHAT	1103	0.397	0.739292	0.01512	0.00297	-7.28833	73.92924	26.07076
PDP2	57546	0.463	0.739617	0.016684	0.025395	-5.429127	73.96166	26.03834
GBP2	2634	0.465	0.740446	0.020686	0.031741	-5.833033	74.04459	25.95541
CPB1	1360	0.398	0.741155	0.024107	0.006565	-10.32755	74.11546	25.88454
PDE4D	5144	0.447	0.741294	0.024777	0.040887	-5.485059	74.12935	25.87065
IL1B	3553	0.513	0.741329	0.024951	0.021821	-11.17805	74.13295	25.86705
C7	730	0.608	0.741463	0.025597	0.008206	-5.73542	74.14634	25.85366
TLE4	7091	0.423	0.742105	0.028695	0.000586	-11.76738	74.21053	25.78947
FBXW2	26190	0.557	0.742667	0.031404	0.010471	-8.787423	74.26667	25.73333
DACH1	1602	0.48	0.743034	0.033177	0.024123	-7.420878	74.30341	25.69659
LEPR	3953	0.527	0.74435	0.039529	0.03392	-5.442258	74.43503	25.56497

RAD18	56852	0.56	0.746667	0.050708	0.039987	-5.300597	74.66667	25.33333
LHB	3972	0.537	0.746871	0.051692	0.056333	-15.47241	74.68707	25.31293
DUSP14	11072	0.478	0.748044	0.057354	0.004704	-27.84778	74.80438	25.19562
NTN1	9423	0.554	0.749662	0.065162	0.03028	-5.673872	74.96617	25.03383
RAPGEF2	9693	0.465	0.75	0.066795	0.008277	-5.318624	75	25
PER1	5187	0.531	0.75	0.066795	0.007479	-5.874209	75	25
NFKBIL1	4795	0.475	0.750395	0.068701	0.028261	-10.65619	75.03949	24.96051
PPP5C	5536	0.47	0.750799	0.070649	0.0292	-6.031089	75.07987	24.92013
STAT6	6778	0.567	0.750993	0.071589	0.006417	-8.875631	75.09934	24.90066
ATP8B2	57198	0.558	0.751009	0.071666	0.045796	-8.211588	75.10094	24.89906
FABP4	2167	0.612	0.751843	0.075688	0.004549	-129.1994	75.18428	24.81572
FLJ23360	65259	0.564	0.752	0.076447	0.021047	-7.959569	75.2	24.8
CGI-105	51011	0.404	0.752328	0.078028	0.012857	-6.847379	75.23277	24.76723
AKR1C2	1646	0.48	0.754717	0.089559	0.028931	-5.011308	75.4717	24.5283
EPOR	2057	0.57	0.754967	0.090765	0.024847	-14.45762	75.49669	24.50331
T	6862	0.481	0.756289	0.097147	0.016604	-5.803154	75.62893	24.37107
CLCNKB	1188	0.572	0.756614	0.098713	0.03697	-5.994753	75.66138	24.33862
TCF19	6941	0.479	0.757911	0.104975	0.004746	-7.674906	75.79114	24.20886
DYT1	1861	0.579	0.759843	0.114295	0.031263	-11.94781	75.98425	24.01575
SAP18	10284	0.386	0.759843	0.114295	0.026441	-10.55408	75.98425	24.01575
CGB8	94115	0.575	0.760582	0.117863	0.020843	-6.414327	76.0582	23.9418
LDHB	3945	0.554	0.760989	0.119828	0.008487	-5.064217	76.0989	23.9011
AVP	551	0.515	0.761271	0.12119	0.008961	-6.512743	76.12712	23.87288
FBXO25	26260	0.572	0.762667	0.127924	0.022229	-30.59745	76.26667	23.73333
APOL1	8542	0.589	0.762953	0.129308	0.006062	-20.45057	76.29534	23.70466
HLA-DMA	3108	0.528	0.763006	0.12956	0.012556	-5.438121	76.30058	23.69942
HCFC2	29915	0.416	0.763303	0.130994	0.125422	-12.23	76.33028	23.66972
ALDOC	230	0.593	0.764175	0.135204	0.028542	-45.20765	76.41753	23.58247
UBE2E3	10477	0.425	0.764388	0.136233	0.007276	-6.563555	76.43885	23.56115
TBCC	6903	0.436	0.764912	0.138761	0.001753	-7.919178	76.49123	23.50877
ADAM15	8751	0.464	0.768212	0.154685	0.040162	-5.238903	76.82119	23.17881
CDH3	1001	0.581	0.768519	0.156165	0.017371	-15.87218	76.85185	23.14815
MYCN	4613	0.435	0.769912	0.162887	0.034612	-5.495328	76.99115	23.00885
EBP	10682	0.441	0.770979	0.168039	0.016045	-5.89609	77.0979	22.9021
RTN4RL2	349667	0.546	0.771186	0.16904	0.027052	-5.048893	77.11864	22.88136
LIPE	3991	0.562	0.771978	0.17286	0.000571	-5.148329	77.1978	22.8022
RAB26	25837	0.439	0.772887	0.177249	0.026686	-6.281148	77.28873	22.71127
TRNT1	51095	0.492	0.773585	0.180615	0.010256	-5.811994	77.35849	22.64151
ACADM	34	0.387	0.774	0.182619	0.020968	-5.800939	77.4	22.6
GAA	2548	0.602	0.774775	0.186358	0.04793	-6.348409	77.47748	22.52252
TAP2	6891	0.493	0.775157	0.188203	0.012385	-5.867651	77.51572	22.48428
MAD2L2	10459	0.565	0.776099	0.192748	0.005487	-47.95611	77.60989	22.39011
PPP1R12C	54776	0.468	0.777409	0.199069	0.051731	-6.896057	77.74086	22.25914
ARHGEF11	9826	0.482	0.777419	0.19912	0.007185	-5.077996	77.74194	22.25806
RARA	5914	0.664	0.777518	0.199594	0.006619	-5.569253	77.75176	22.24824
ACP1	52	0.497	0.777778	0.20085	0.003439	-19.31143	77.77778	22.22222

PPP6C	5537	0.487	0.777955	0.201707	0.032754	-10.37699	77.79553	22.20447
USP33	23032	0.5	0.778816	0.205861	0.031455	-5.963971	77.88162	22.11838
TP53AP1	11257	0.486	0.78135	0.218092	0.05769	-5.959153	78.13505	21.86495
CHRNE	1145	0.522	0.781437	0.21851	0.032048	-7.144744	78.14371	21.85629
CPNE5	57699	0.643	0.782238	0.222377	0.017141	-9.966657	78.22384	21.77616
LEP	3952	0.563	0.783032	0.226207	0.06461	-10.89042	78.3032	21.6968
AKNA	80709	0.473	0.783113	0.226596	0.021467	-5.950619	78.31126	21.68874
PCDHA6	56142	0.6	0.78329	0.227451	0.031897	-5.273714	78.32898	21.67102
WBSCR21	83451	0.474	0.784768	0.234586	0.035346	-8.476904	78.47682	21.52318
CA2	760	0.645	0.786585	0.243355	0.010916	-10.96199	78.65854	21.34146
GYS2	2998	0.461	0.786689	0.243858	0.009931	-5.473889	78.66894	21.33106
ID3	3399	0.51	0.787645	0.248468	0.012231	-7.297298	78.76448	21.23552
KIF20A	10112	0.504	0.788732	0.253717	0.009207	-7.764823	78.87324	21.12676
SLC11A2	4891	0.635	0.789801	0.258874	0.009326	-10.62174	78.9801	21.0199
HERC3	8916	0.508	0.791277	0.265999	0.057671	-12.13137	79.12773	20.87227
SETDB1	9869	0.534	0.795827	0.287956	0.033253	-11.64844	79.58271	20.41729
P4HA1	5033	0.48	0.79602	0.288886	0.071152	-8.06574	79.60199	20.39801
MPHOSPH 1	9585	0.511	0.799687	0.306584	0.006093	-72.21627	79.9687	20.0313
RABGGTB	5876	0.445	0.80036	0.30983	0.006162	-6.846299	80.03597	19.96403
USP7	7874	0.516	0.803738	0.326136	0.082304	-7.913914	80.37383	19.62617
ESRRBL1	55081	0.655	0.804668	0.330624	0.017101	-5.26322	80.46683	19.53317
LCN1	3933	0.61	0.804749	0.331015	0.034559	-8.516612	80.47493	19.52507
ADAMTS1 3	11093	0.499	0.804839	0.331446	0.005699	-11.65312	80.48387	19.51613
PDE6G	5148	0.499	0.804839	0.331446	0.006292	-5.818882	80.48387	19.51613
BIRC8	112401	0.604	0.805333	0.333833	0.032989	-11.00079	80.53333	19.46667
PGAM1	5223	0.487	0.807629	0.34491	0.059559	-10.82319	80.76285	19.23715
GLUD1	2746	0.614	0.807895	0.346194	0.006716	-6.528863	80.78947	19.21053
SLC12A2	6558	0.65	0.808458	0.348911	0.013809	-5.694578	80.84577	19.15423
PPP2R2A	5520	0.549	0.808542	0.349318	0.062675	-17.05779	80.8542	19.1458
WWOX	51741	0.55	0.810015	0.356425	0.063698	-14.98905	81.00147	18.99853
ALG6	29929	0.629	0.810567	0.359091	0.024372	-5.511589	81.0567	18.9433
CLN8	2055	0.614	0.812169	0.366823	0.052614	-6.808667	81.21693	18.78307
HGF	3082	0.504	0.812903	0.370365	0.001193	-9.305292	81.29032	18.70968
CDCA7	83879	0.615	0.813492	0.373207	0.03056	-6.068352	81.34921	18.65079
UQCRRF51	7386	0.493	0.816225	0.386397	0.023584	-8.946435	81.62252	18.37748
LTBP2	4053	0.567	0.818182	0.39584	0.013534	-5.055603	81.81818	18.18182
RLF	6018	0.416	0.818898	0.399294	0.041653	-9.390234	81.88976	18.11024
WAS	7454	0.512	0.825806	0.432636	0.003058	-7.794373	82.58065	17.41935
FLJ39616	51275	0.628	0.826316	0.435094	0.004467	-13.27442	82.63158	17.36842
POU3F3	5455	0.42	0.826772	0.437294	0.026036	-5.409582	82.67717	17.32283
HIP1	3092	0.629	0.827632	0.441444	0.000183	-5.926172	82.76316	17.23684
SFRS8	6433	0.524	0.829114	0.448598	0.014665	-5.405221	82.91139	17.08861
AGTRAP	57085	0.629	0.829815	0.451983	0.021326	-5.241184	82.98153	17.01847
PCNA	5111	0.501	0.832226	0.463617	0.075424	-12.79017	83.22259	16.77741
MYH8	4626	0.516	0.832258	0.463772	0.007451	-6.796569	83.22581	16.77419

FDXR	2232	0.483	0.832759	0.466187	0.021142	-13.80941	83.27586	16.72414
EYA1	2138	0.635	0.833333	0.468961	0.053554	-6.592826	83.33333	16.66667
SLAMF6	114836	0.564	0.833703	0.470744	0.014024	-6.891906	83.37029	16.62971
CAP1	10487	0.688	0.839024	0.496426	0.020792	-9.236182	83.90244	16.09756
PDE7B	27115	0.51	0.845771	0.528986	0.107156	-6.356863	84.57711	15.42289
AMFR	267	0.582	0.84593	0.529754	0.021457	-15.96102	84.59302	15.40698
HEXA	3073	0.496	0.846416	0.5321	0.016509	-10.13314	84.64164	15.35836
TCTA	6988	0.483	0.847368	0.536694	0.003509	-5.810939	84.73684	15.26316
GABPA	2551	0.462	0.847706	0.538325	0.263386	-6.278929	84.77064	15.22936
PPP1R16A	84988	0.531	0.848243	0.540914	0.050625	-21.89552	84.82428	15.17572
SERPING1	710	0.527	0.85	0.549394	0.004723	-7.818861	85	15
SLC22A18	5002	0.571	0.850969	0.554069	0.057191	-5.725697	85.09687	14.90313
ALDH3A2	224	0.661	0.851804	0.558101	0.043471	-6.124661	85.18041	14.81959
SLAMF1	6504	0.729	0.85363	0.566913	0.087599	-5.339571	85.363	14.637
CISH	1154	0.647	0.85582	0.577482	0.058225	-6.522036	85.58201	14.41799
YAP1	10413	0.483	0.856383	0.580199	0.091147	-8.575682	85.6383	14.3617
USP12	219333	0.55	0.856698	0.581718	0.17408	-6.209265	85.66978	14.33022
KCNH1	3756	0.551	0.866352	0.62831	0.039556	-5.551057	86.63522	13.36478
OPA1	4976	0.54	0.870968	0.650585	0.012516	-5.667253	87.09677	12.90323
P4HB	5034	0.529	0.87728	0.681049	0.177086	-35.83085	87.72803	12.27197
DARS	1615	0.67	0.879265	0.690628	0.083947	-5.642411	87.92651	12.07349
TYR	7299	0.49	0.881295	0.700424	0.029622	-5.512439	88.1295	11.8705
RAB40A	142684	0.503	0.885563	0.721023	0.079438	-5.356819	88.55634	11.44366
KIAA1181	57222	0.576	0.888889	0.737072	0.361858	-6.414327	88.88889	11.11111
SAP30	8819	0.452	0.889764	0.741294	0.073777	-6.048819	88.97638	11.02362
RORA	6095	0.766	0.896956	0.776001	0.114561	-5.113393	89.69555	10.30445
CCL7	6354	0.691	0.923797	0.905537	0.113918	-5.620717	92.37968	7.620321
PBX3	5090	0.631	0.929308	0.932134	0.230413	-10.46488	92.93078	7.069219
CDC37L1	55664	0.709	0.932895	0.949444	0.008815	-12.03133	93.28947	6.710526
RAB6A	5870	0.639	0.94109	0.988994	0.334321	-6.854465	94.10898	5.891016