Can interleukin-6 promote human colorectal cancer progression through changes in microRNA expression?

By

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Abstract

Colorectal cancer (CRC) is the third most common cancer worldwide with over a million new cases each year. Eighty percent of CRC cases have no genetic predisposition and result from a series of epigenetic and genetic alterations, referred to as 'sporadic' CRC. Diets rich in red or processed meats (containing dietary carcinogens) as well as bowel inflammation are a major risk factors for disease, however the underlying mechanisms are not yet well understood. Upregulation of pro-inflammatory cytokines and cytochrome P450 (CYP450) enzymes that activate dietary carcinogens as well as microRNA (miRNA) dysregulation have been observed in CRC tissue. Interleukin-6 (IL6) is a multifunctional cytokine thought to play numerous roles in tumour progression including activation of tumour-promoting signalling pathways, epigenetic gene regulation and alteration of DNA repair mechanisms; thus presence of IL6 may be key to promoting CRC tumour progression. I hypothesised that elevated levels of IL6 in the tumour microenvironment (TME) could alter miRNA expression and induce local activation of dietary carcinogens, thereby stimulating epigenetic and genetic changes that promote CRC. The current project investigated this hypothesis using a mechanistic approach with *in vitro* cell culture techniques. Phenotypic changes in response to IL6 treatment were observed and the underlying causes were determined by performing genetic and epigenetic studies. The findings from this project identified three potential mechanisms of IL6-mediated CRC promotion, which involve miRNAs, STAT3 signalling and DNA methylation. First, IL6 promoted dietary carcinogen-mediated DNA damage by inducing CYP450 expression. Second, IL6 stimulated CRC cell proliferation, migration and invasion through gene and miRNA

expression changes. Third, IL6- mediated CRC-immune cell crosstalk in the TME resulting in the maintenance of IL6 secretion. Taken together, these data suggest that IL6 plays multiple roles in promoting CRC. Understanding these molecular events could lead to better prevention and therapeutic strategies.

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List of abbreviations

Abbreviation	Meaning
2D	Two-Dimensional
3D	Three-Dimensional
7-ER	7-ethoxyresorufin
AhR	Aryl Hydrocarbon Receptor
ANOVA	One-Way Analysis of Variance
APC	Adenomatous Polyposis Coli
BaP	Benzo[a]pyrene
ChIP	Chromatin Immunoprecipitation
CAC	Colitis-associated CRC
CAF	Cancer-Associated Fibroblast
CRC	Colorectal Cancer
CSC	Cancer Stem Cell
CTSD	Cathepsin D
CYP	Cytochrome P450
DNA	Deoxyribonucleic Acid
DNMT1	DNA Methyltransferase 1
dNTP	Deoxyribonucleotide Triphosphate
ECM	Extracellular Matrix
ELISA	Enzyme-Linked Immunosorbent Assay
EMT	Epithelial-to-Mesenchymal Transition
EROD	ethoxyresorufin-O-deethylase
FBS	Foetal Bovine Serum

Abbreviation	Meaning
HDAC	Histone Deacetylases
IBD	Inflammatory Bowel Disease
IL	Interleukin
IL6R	IL6 receptor
JAK	Janus Tyrosine Kinase
LPS	Lipopolysaccharides
miRNA	MicroRNA
MMP	Matrix Metalloproteinase
MN	Micronucleus
mRNA	messenger RNA
NK	Natural Killer cells
NSAIDs	Non-Steroidal Anti-Inflammatory Drugs
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
qPCR	quantitative-Polymerase Chain Reaction
RISC	RNA-Induced Silencing Complex
ROS	Reactive oxygen species
RNA	Ribonucleic Acid
SEM	Standard Error of the Mean
sIL6R	soluble IL6R
SOCS3	Suppressor Of Cytokine Signalling 3
STAT3	Signal Transducers and Activators of Transcription 3
TAM	Tumour Associated Macrophage

Abbreviation	Meaning
TGFβ	Transforming Growth Factor beta
Th	T helper cell
TLR	Toll-Like Receptor
TME	Tumour Microenvironment
TNFα	Tumour Necrosis Factor alpha
Treg	Regulatory T cells
UTR	Untranslated Region
VEGF	Vascular Endothelial Growth Factor

Statement of originality

I hereby affirm that the work presented in this thesis represents my own original work, unless otherwise stated. All other work has been appropriately referenced. I confirm that this thesis has been composed by myself and has not been previously submitted to any other institution for any degree.

I have included parts of this thesis in the following peer-reviewed research publications:

Patel, S.A.A. and Gooderham, N.J., 2015. Interleukin-6 mediates crosstalk between immune and cancer cells *via* miR21 and miR29b. *Molecular Cancer Research* (In Press).

Patel, S.A.A. and Gooderham, N.J., 2015. Interleukin-6 promotes dietary carcinogen-induced DNA damage in colorectal cancer cells. *Toxicology Research*, 4, pp.858-66.

Patel, S.A.A. *et al.*, 2014. Interleukin-6 mediated upregulation of CYP1B1 and CYP2E1 in colorectal cancer involves DNA methylation, miR27b and STAT3. *British journal of cancer*, 111(12), pp.2287-96.

Copyright declaration

'Il faut avoir de la persévérance, et surtout de la confiance en soi. Il faut croire que l'on est doué pour quelque chose, et que, cette chose, il faut l'atteindre coûte que coûte.'

'We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something, and that this thing, at whatever cost, must be attained.'

Marie Curie

(Letter to brother, 1894)

Dedication

To people who have fought or are still fighting a debilitating disease such as cancer,

To my Grandmother, who lost her fight,

To my parents, who made me believe that I was gifted for something and always encouraged me to pursue my ambitions.

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- Chapter 1 -

Introduction

1.1 Colorectal cancer, a lifestyle disease?

Colorectal cancer (CRC) is the term used to describe a cancer originating in the epithelium lining of the large intestine, which is divided into two parts: colon and rectum (Figure 1.1 A). The large intestine is the final part of the digestive tract measuring approximately 1.5 meters in the human adult. Its role is to absorb vitamins and water from solid food waste prior to excreting the unwanted excess as stools. The epithelium lining of the large intestine is composed of a series of crypts each containing stem cells at their base, which divide and differentiate while moving along the crypt into various epithelial cell types: colonocytes, mucous-secreting goblet cells, peptide hormone-secreting endocrine cells and the antibacterial protein-secreting Paneth cells (Figure 1.1 B). Once at the top of the crypt, these differentiated cells undergo apoptosis and are shed by the colon. On average, $1x10^{10}$ colon epithelial cells are discarded each day and to replace these, transient cells must proliferate rapidly, thus increasing the possibility for cell division errors leading to mutations and higher risk of cancer development (Humphries & Wright 2008; Tomasetti & Vogelstein 2015). An accumulation of these events is thought to result in aberrant cell growth and tumour formation.

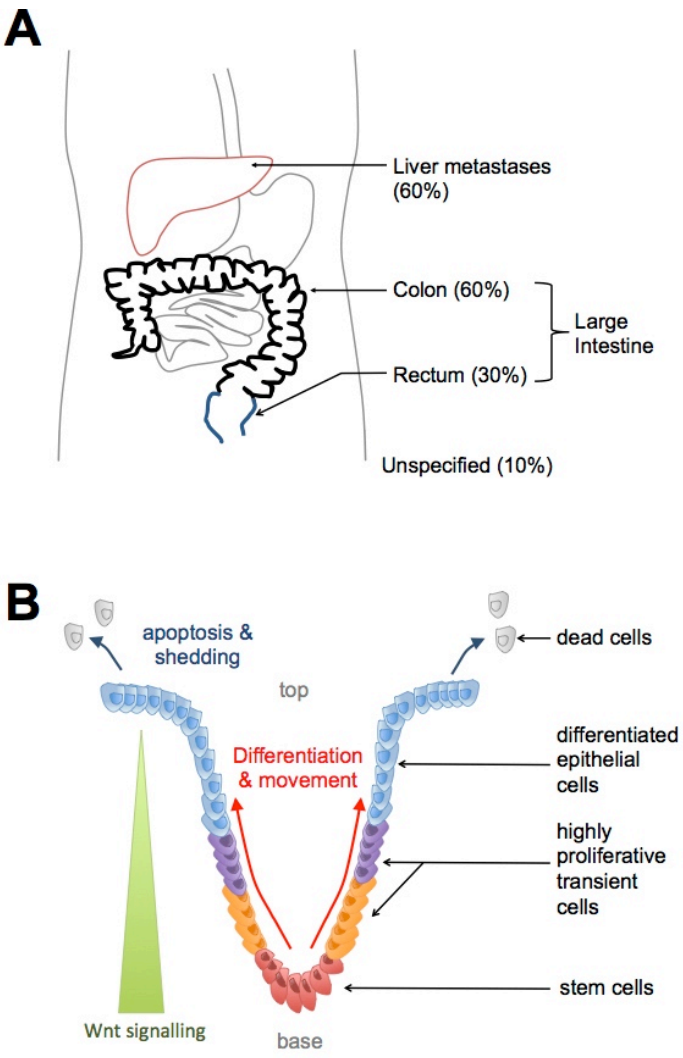

Figure 1.1 Large intestine and crypt architecture. (A) Diagram of large intestine with percentage of the colorectal cancer case occurrence at each site, adapted from Cancer Research UK, 2014. (B) Colonic crypt structure, adapted from Humphries & Wright 2008.

CRC is the third most common cancer worldwide; occurrence rates are highest in Australia/New Zealand while Western Africa has the lowest rates (Midgley & Kerr 1999). Migration from low occurrence countries to countries with a Western culture increases incidence rates. CRC thus appears to be linked to Western culture, suggesting lifestyle plays an important role in disease risk (Haggar & Boushey 2009). In the UK alone, around 41,000 new cases of CRC were diagnosed in 2011 with a male to female ratio of 13:10, making it the fourth most common cancer in the country. The disease accounts for 10% of all cancer

deaths in the UK and 8% worldwide (Cancer Research UK 2014), and thus presents a major global health burden. As for the majority of cancers, early diagnosis is key to patient survival. While 5-year survival rate for local stage disease is high (90%), this rate drops to 12% when disease has metastasised (Cancer Research UK 2014). More importantly, overall CRC survival rates have not seen any significant improvement in the last 20 years highlighting the need for better preventive measures and diagnosis techniques, which currently rely on colonoscopies and faecal occult tests. Further understanding of the specific mechanisms involved in carcinogenesis is key to developing novel strategies to improve patient outcome.

Most cases of CRC (around 90%) start off as a benign growth on the intestinal wall called a polyp or adenoma, which if left untreated can become cancerous over time through a series of genetic and epigenetic changes, a process known as carcinogenesis (Figure 1.2). In the multi-step model proposed by Vogelstein and co-workers (Fearon & Vogelstein 1990), cellular transformation from normal to malignant requires a succession of changes in different gene activities, each conferring a selective advantage to the transformed cell resulting in clonal expansion and tumour formation. Most human cancers are thought to be caused by 2-8 changes that develop over 20-30 years (Vogelstein *et al.* 2013). Genes involved in this process regulate key cellular events such as division, DNA repair and apoptosis. These can either be aberrantly activated (conversion of proto-oncogenes to oncogenes) or inappropriately silenced (tumour-suppressor genes) depending on the functional outcome of their deregulation. Regulation of these gene activities may occur by point-mutation, gene amplification, chromosome loss or epigenetic mechanisms.

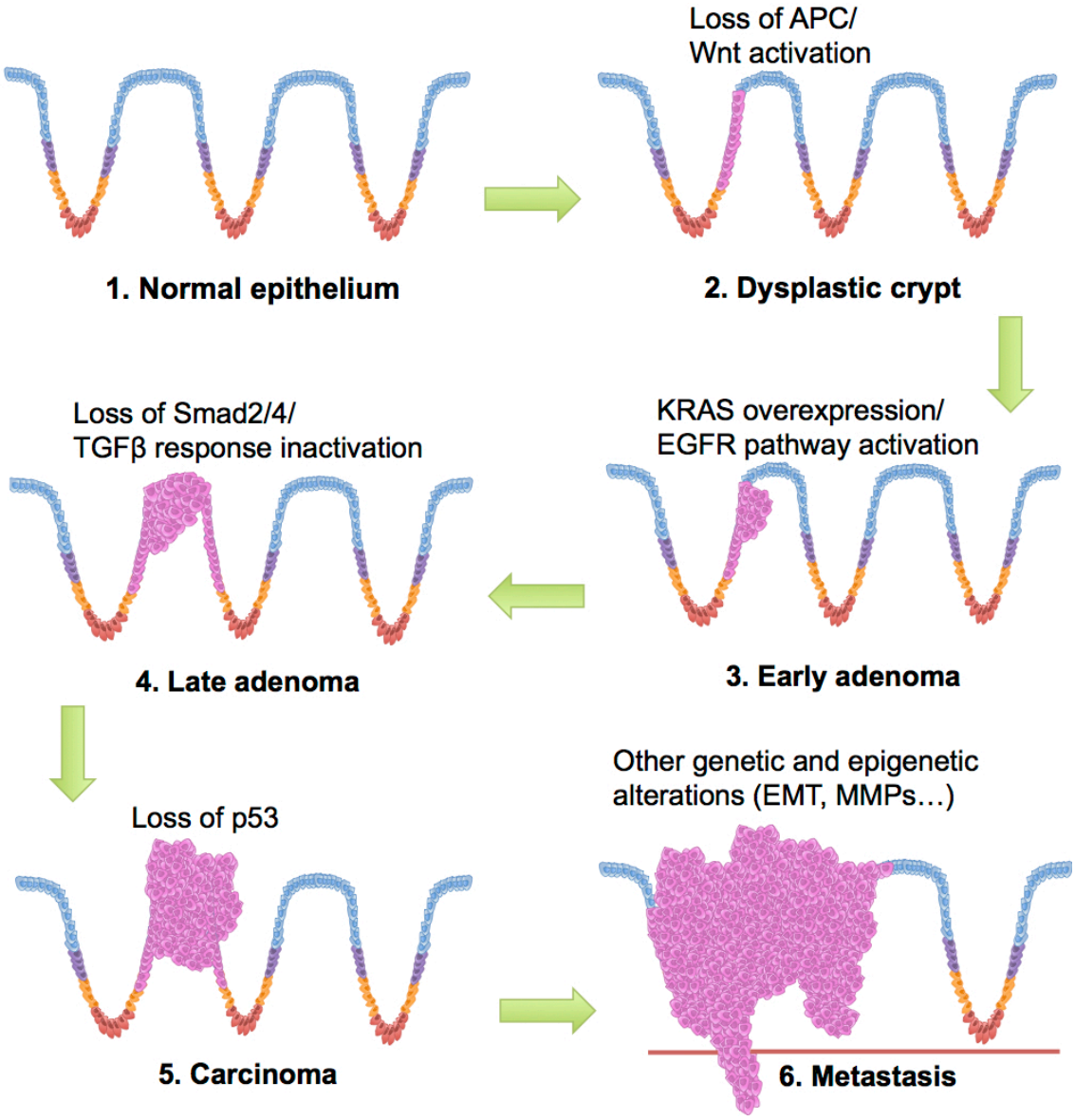

Figure 1.2 Vogelstein's multi-step genetic model for sporadic colorectal cancer development. *APC* mutation resulting in Wnt activation in transient proliferating cells leads to dysplasia. Further genetic alterations in *KRAS* and *Smad2/4* leads to further adenoma growth. Loss of p53 function results in carcinoma and further genetic changes leads to tumour metastasis. Adapted from Rajagopalan, Nowak, Vogelstein, & Lengauer 2003.

The inactivation of tumour-suppressor genes is believed to predominate over the activation of oncogenes (Fearon & Vogelstein 1990). Indeed, an early event in colorectal tumourigenesis is thought to be the loss-of-function mutation of the *adenomatous polyposis coli* (*APC*) gene (in 85% of cases), which leads to the formation of polyps. Further alterations are required for the polyps to

progress to malignancy. These include activation of the *KRAS* oncogene and *p53* tumour-suppressor gene inactivation through mutation. *KRAS* mutation in a single allele suffices to promote activity and appears in 50% of CRC tumours (Hanahan & Weinberg 2000; Fearon & Vogelstein 1990), while *p53* mutation confers resistance to apoptosis (Hollstein *et al.* 1991; Fearon & Vogelstein 1990). The latter occurs not only in CRC but also in the majority of solid tumours (Hollstein *et al.* 1991). This multi-step genetic model for CRC initiation was first established by the pioneering works of Professor Bert Vogelstein and colleagues in the 1980s (Fearon & Vogelstein 1990; Cho & Vogelstein 1992; Cummins *et al.* 2006). Once established, the primary tumour is thought to undergo further genetic and epigenetic alterations resulting in tumour progression and metastasis (Fearon & Vogelstein 1990). More recently, a study by Tomasetti and Vogelstein demonstrated a strong positive correlation between number of stem cell divisions in a particular tissue and lifetime risk of cancer in that tissue, suggesting that the majority of mutations required for cancer development result from errors in DNA replication, with the rest of the mutations being caused by hereditary or environmental factors (Tomasetti & Vogelstein 2015).

Risk factors for CRC are various and genetic predisposition accounts for only 6% of cases (Rustgi 2007). The two major subtypes of hereditary CRC are hereditary non-polyposis colorectal cancer (also known as Lynch syndrome) and familial adenomatous polyposis, each caused by germline mutations in genes involved in the DNA mismatch-repair pathway and *APC* respectively (Midgley & Kerr 1999). However, the vast majority of CRC cases arise sporadically. While DNA replication errors are the primary source of *de novo* or non-inherited genetic changes responsible for cancer (Tomasetti & Vogelstein

2015), environmental factors also contribute to generating mutations in the cells lining the colorectal epithelial wall (Fearon & Vogelstein 1990). An astounding 90% of CRC cases occur in individuals aged above 50 years (Haggar & Boushey 2009), highlighting age as a key factor for disease, which supports Vogelstein's multi-step progressive model for colorectal carcinogenesis. Nonetheless, other risk factors for disease linked to lifestyle are modifiable such as lack of physical activity, obesity with abdominal fatness, type 2 diabetes, cigarette smoking, and most importantly dietary habits (Tenesa & Dunlop 2009), thus making disease prevention possible, at least in theory.

Diets rich in animal fat and processed meat are thought to strongly promote development of CRC as well as high consumption of alcohol, while dietary fibre and fruit/vegetable intake are believed to prevent disease (Midgley & Kerr 1999; Chao *et al.* 2005). In addition, it is estimated that changes in nutritional practices could reduce CRC incidence by up to 70% (Haggar & Boushey 2009), further highlighting the critical contribution of diet to disease risk and the potential to exploit this understanding for preventing disease.

Diet may also contribute to colonic inflammation, another important risk factor for disease. Western-style diets, characterised by high-fat and low fibre content, have been shown to induce oxidative stress, macrophage recruitment and secretion of inflammatory proteins in the colon (Erdelyi *et al.* 2009). Furthermore, individuals with a chronic bowel inflammation such as inflammatory bowel disease (IBD) including ulcerative colitis and Crohn's disease are 70% more susceptible to getting CRC than healthy individuals (Feagins *et al.* 2009). This risk is increased with duration of IBD and extent of inflammation. Mechanisms underlying the link between IBD and development

of CRC remain unclear. However, production of reactive oxygen species and increased levels of pro-inflammatory cytokines are thought to be involved with aberrant interleukin-6 (IL6) signalling playing a key role in IBD pathogenesis (Scheller *et al.* 2006; Li *et al.* 2010). This is evidenced through reports of antibody-mediated inhibition of IL6 being sufficient to suppress chronic colonic inflammation in Crohn's disease (Ito *et al.* 2004). IL6 has also been implicated in promoting cancer development and progression, further corroborating its potential involvement in sporadic CRC.

Interestingly, non-steroidal anti-inflammatory drugs (NSAIDs) such as aspirin have been reported to reduce CRC risk by 40-50% (Smalley *et al.* 1999; Williams *et al.* 1997). More recently, large-scale epidemiological studies led by Professor Peter Rothwell have reported that daily intake of aspirin not only decreases the risk of cancer development, it also reduces the risk of metastasis and cancer-related mortality (Rothwell *et al.* 2011; Rothwell, Wilson, *et al.* 2012; Rothwell, Price, *et al.* 2012; Algra & Rothwell 2012). Importantly, these benefits were not only observed with long-term aspirin use, but were also detected as from 3 years onward, demonstrating that short-term use can be beneficial as well (Rothwell, Price, *et al.* 2012). Furthermore, these studies found that CRC risk was the most markedly reduced by daily aspirin intake (Rothwell, Wilson, *et al.* 2012; Algra & Rothwell 2012), further highlighting the importance of inflammation in CRC development.

Therefore, even though a multitude of factors are involved in CRC development, environment does appear to play a significant role. Importantly, some of the lifestyle-linked factors are preventable, however prevention in order to be effective requires a profound understanding of the mechanisms involved.

1.2 Dietary carcinogens and cytochrome P450 enzymes, mediators of CRC carcinogenesis?

As the colon is part of the digestive system, it is particularly susceptible to carcinogens present in the diet and over the last few decades, numerous epidemiological studies have reported a significant correlation between high consumption of red and processed meats, known sources of dietary carcinogens, to increased incidence of CRC (Chao *et al.* 2005). The method of cooking the meats, such as frying at high temperatures and formation of a heavily browned surface, has also been shown to increase risk of CRC. Commonly occurring dietary carcinogens include benzo[a]pyrene (BaP) and 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (Creton *et al.* 2007; Irigaray *et al.* 2006). These compounds are considered carcinogenic due to their ability to form DNA adducts, resulting in DNA damage and mutations. They are known as pro-carcinogens as they require metabolic activation to their respective DNA-damaging agents by cytochrome P450 (CYP) enzymes (Figure 1.3; Gooderham *et al.* 2007; Lodovici *et al.* 2004).

BaP is a five-ring polycyclic aromatic hydrocarbon generated during the combustion of organic compounds and is commonly found in motor fumes, cigarette smoke and cooked meats (Lodovici *et al.* 2004). It is thought to be involved in tobacco- and diet-associated cancers due to its mutagenic potential. Like many carcinogens, it is activated into its genotoxic compound, 7,8-diol-9,10-epoxy BaP, by CYP1 A1, A2 and B1 (Figure 1.3). The activated molecule covalently binds DNA at the N2 atoms in guanine residues thus disrupting the double-helical structure, which results in DNA damage including double-strand breaks, deletions and points mutations (Lodovici *et al.* 2004; Trushin *et al.*

2012). Furthermore, BaP exposure has been shown to induce an inflammatory response as well as promote cell proliferation through gene expression changes (Albert *et al.* 1996; Volkov & Kobliakov 2011; Ouyang *et al.* 2007; Hockley *et al.* 2007). It is thought that 7,8-diol-9,10-epoxy BaP can alter gene expression by activating a number of transcription factors including NFκB, AP1 and the aryl hydrocarbon receptor (AhR) pathway (Ouyang *et al.* 2007; Hockley *et al.* 2007).

PhIP is the most abundant heterocyclic amine found primarily in meats cooked at high temperatures, particularly fish, chicken and beef (Murray *et al.* 1993). As with BaP, it is activated by CYP1 A1, A2 and B1 enzymes into its mutagenic compound, N-hydroxy PhIP (Figure 1.3; Gooderham *et al.* 2001; Buonarati & Felton 1990). N-hydroxy PhIP can then be esterified by sulphur transferase or acetyl transferase. This activated PhIP can intercalate DNA and covalently bind the C8 atom in guanine residues resulting in DNA adducts and mutations (Buonarati & Felton 1990). Interestingly, epidemiological studies have found correlations between meat consumption, PhIP intake, and colon, breast and prostate cancers (Chao *et al.* 2005; Sinha *et al.* 2005; Cross *et al.* 2005; Sinha *et al.* 2000), and PhIP-DNA adducts have been detected in these tissues (Tang *et al.* 2007; Malfatti *et al.* 2006; Zhu *et al.* 2003), supporting its role as a mutagen in these tissue types. It has also been shown to induce breast cancer cell invasion (Lauber & Gooderham 2011) and have oestrogenic activity (Papaioannou *et al.* 2014; Lauber *et al.* 2004). PhIP can also activate tumour-promoting signalling pathway MAPK/ERK, resulting in increased cell proliferation and migration (Creton *et al.* 2007; Gooderham *et al.* 2007; Lauber *et al.* 2004; Lauber & Gooderham 2007).

While the ability for these dietary carcinogens to induce DNA damage is thought to be primarily associated with cancer initiation stages, their ability to alter key cellular signalling pathways is linked to promoting tumour progression (Ouyang *et al.* 2007). Therefore, these compounds are involved in all cancer stages.

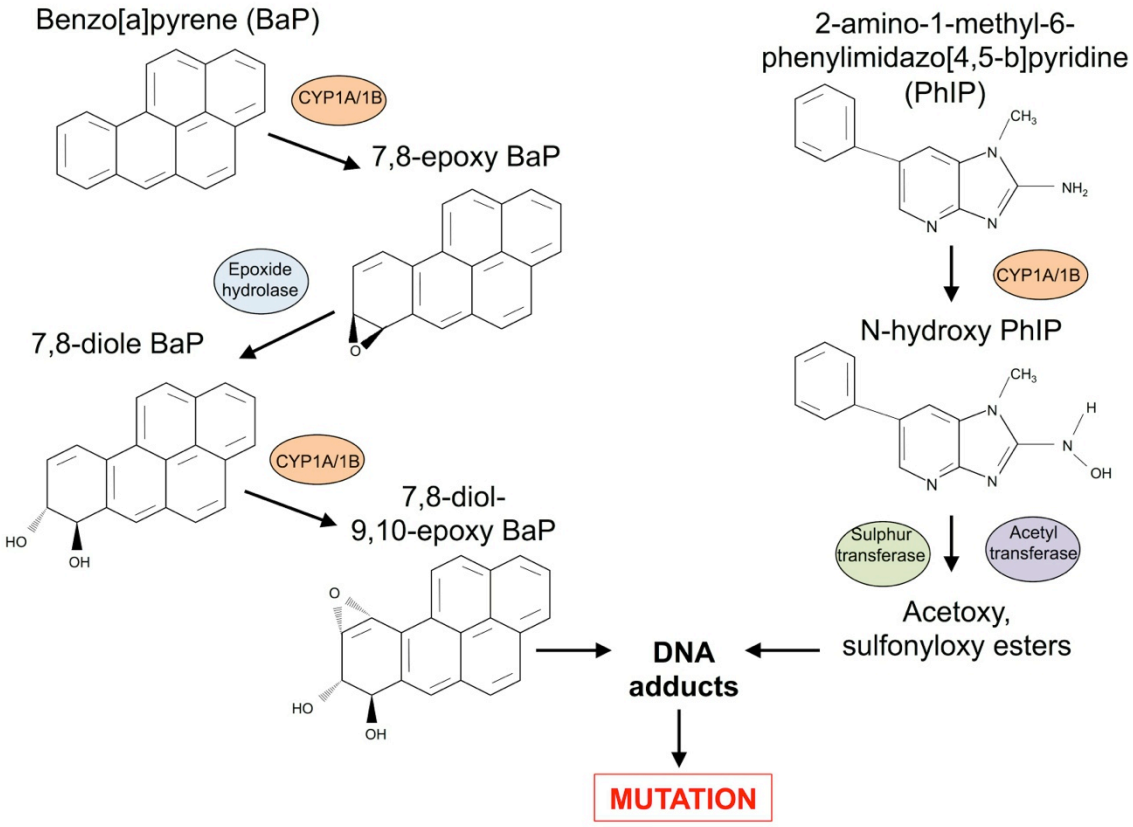

Figure 1.3 Dietary pro-carcinogens BaP and PhIP activation pathways. BaP is metabolised by CYP1A/1B to generate 7,8-epoxy BaP, which is converted into 7,8-diole BaP. The latter is again metabolised by CYP1A/1B to form the genotoxic product 7,8-diol-9,10-epoxy BaP. CYP1A/1B enzymes metabolise PhIP into N-hydoxy PhIP, which is converted into acetoxy and sulphonyloxy esters by sulphur/acetyl transfecrases. Adapted from Gooderham *et al.* 2001 and Trushin *et al.* 2012.

The CYP450 superfamily of enzymes is subdivided into 18 families. There are 57 active *CYP450* genes in the human genome (Sim & Ingelman-Sundberg 2010). CYP450 enzymes catalyse oxidative reactions and display a broad substrate specificity allowing them to metabolise a variety of endogenous substrates, drugs and xenobiotics resulting in either their bioactivation or deactivation. The first three families (CYP1-3) are responsible for metabolising exogenous chemicals, whereas the other CYP450 families generally metabolise endogenous substrates (Sim & Ingelman-Sundberg 2010). While most CYP450s are primarily expressed in the human liver, CYP2E1, CYP1B1 and CYP1A1 (known to activate dietary carcinogens) are found in CRC tissue (Patel *et al.* 2014; McFadyen *et al.* 2004; Murray *et al.* 1997). CYP450 enzymes have been implicated in cancer development and progression and are overexpressed in a variety of cancers including CRC (Murray *et al.* 1997; Gooderham *et al.* 2007; Braeuning *et al.* 2011; Kasai *et al.* 2013; Rodriguez & Potter 2013), suggesting they play an important role in CRC carcinogenesis.

1.3 The tumour microenvironment and its role in CRC progression.

Tumours are surrounded by a variety of non-tumour cell types, which secrete signalling molecules into the tumour microenvironment (TME) including growth factors, chemokines and cytokines that activate key transcription factors to promote tumour progression. These non-tumour cells are genetically stable compared to cancer cells and are thus less likely to acquire resistance to therapy, making them attractive therapeutic targets (Mbeunkui & Johann 2009). Understanding the complex intercellular interactions within the microenvironment is crucial for the development of cancer therapies that target the TME.

Tumours are thought to reorganise their extracellular matrix (ECM) by secreting ECM degrading proteinases to allow tumour cells to proliferate and invade surrounding tissues (Lu *et al.* 2012; Pickup *et al.* 2014). The ECM is a complex network of proteins including proteoglycans and fibrous proteins such as collagen (Alberts *et al.* 2002). The composition of these different elements varies depending on tissue type and defines tissue structure. Organisation of the ECM is an important feature of the TME and ECM stiffness is characteristic of tumours (Lu *et al.* 2012). Its plasticity means it can readily be reorganised by tumour-secreted proteins, and as it also acts as a barrier for cell invasion, its remodelling is an essential step in cancer growth and metastasis (Lu *et al.* 2012; Pickup *et al.* 2014). Proteins that mediate this remodelling are secreted by tumour cells and include matrix metalloproteinases (MMPs), cathepsins and sulphatases (Azzam *et al.* 1993; Mott & Werb 2004; Gonzalez-Villasana *et al.* 2015; Che *et al.* 2015; Yang *et al.* 2011; Mohamed & Sloane 2006). These proteinases can also target non-ECM proteins such as growth factors and

cytokines making their role within the microenvironment complex and versatile (Kessenbrock *et al.* 2010; Mbeunkui & Johann 2009).

In addition, tumour cells secrete a variety of growth factors into the microenvironment including osteopontin, galectin-3 and TGFβ (Mbeunkui & Johann 2009). Osteopontin and galectin-3 are associated with metastatic disease in a variety of cancer types including CRC (Wu *et al.* 2013; Mole *et al.* 2011), while TGFβ is known to inhibit early-stage CRC tumour growth but promote advanced CRC metastasis (Roberts & Wakefield 2003). TGFβ secretion by cancer cells also promotes recruitment of anti-inflammatory immune cells to tumour site, as well as activates adjacent fibroblasts, which differentiate into cancer-associated fibroblasts (CAFs) and promote tumourigenesis (Yeung *et al.* 2013; Hawinkels *et al.* 2014; Calon *et al.* 2014).

Fibroblasts are the major cell-type of the stroma and when differentiated into CAFs, they are crucial for tumour progression and metastasis of most cancer types including CRC (Mbeunkui & Johann 2009; Kalluri & Zeisberg 2006; Tommelein *et al.* 2015). In fact, CAFs have a variety of functions within the TME: they promote wound healing and control inflammation by regulating immune cell infiltration through cytokine and chemokine release such as TGFβ, IL6, IL1 and CCL2; they play an important role in ECM remodelling, as they secrete MMPs and contribute to ECM deposition by producing collagen (Mbeunkui & Johann 2009; Cirri & Chiarugi 2011); they are also involved in the epithelial-to-mesenchymal transition (EMT) of tumour cells, which promotes their ability to invade surrounding tissues, as well as angiogenesis, the formation of new blood vessels essential for tumour growth (Mbeunkui & Johann 2009; Cirri & Chiarugi 2011).

Rapid tumour growth results in hypoxic conditions in the TME, which triggers vascular endothelial growth factor (VEGF) release by pericytes, CAFs and tumour cells (Bhome *et al.* 2015). VEGF induces angiogenesis by promoting endothelial cell migration to the hypoxic regions and generation of new blood vessels. VEGF is also overexpressed in CRC tissue and expression correlates with patient survival (Cao *et al.* 2009), thus its presence in the TME appears to be important for CRC tumour progression.

Once tumours have formed, they secrete chemokines and cytokines such as IL8, CCL2, IL6 and TNFα, which recruit innate and adaptive immune cells to the tumour site (Bhome *et al.* 2015; Vesely *et al.* 2011). These cells can have pro- and anti-tumour effects depending on their nature. Immunosurveillance mechanisms instigated by M1 macrophages, T-helper 1 (Th1) cells, cytotoxic T cells and Natural Killer cells (NK) can inhibit tumour progression, but tumours are able to manipulate the TME in order to evade this anti-tumour immune response by recruiting anti-inflammatory immune cells such as M2 macrophages, regulatory T cells (Treg) and Th2 cells (Bhome *et al.* 2015; Vesely *et al.* 2011).

Macrophages are a major component of the TME, and in some cases, they can represent more than 50% of the tumour mass (Lewis & Pollard 2006). Increased macrophage density is generally associated with poor prognosis (Tsutsui *et al.* 2005; Hamada *et al.*; Hanada *et al.* 2000; Mäkitie *et al.* 2001); however, in CRC, the amount of macrophage infiltrate correlates with a favourable prognosis (Zhou *et al.* 2010; Forssell *et al.* 2007). This may be due to the fact that macrophages are a very heterogenic population and depending on their nature, they can have different functions in tumourigenesis. The two major

subtypes are M1 and M2 macrophages. M1 cells are activated by microbial products and initiate a pro-inflammatory response by secretion of pro-inflammatory cytokines such as TNFα, generation of reactive oxygen species (ROS) and cytotoxic activity against the cancer cells. M2 or 'alternatively activated' cells are generally immunosuppressive through release of anti-inflammatory cytokines such as IL10, and are thought to promote tumour progression (Noy & Pollard 2014; Bhome *et al.* 2015; Balkwill & Mantovani 2012; Mantovani & Sica 2010; Solinas *et al.* 2009). Profiling experiments on tumour-associated macrophages (TAMs) have demonstrated that the TAM population is very diverse and composed of various different subtypes but are more inclined toward an M2-like tumour-promoting phenotype (Ojalvo *et al.* 2009; Ojalvo *et al.* 2010). Each subtype appears to be uniquely suited to perform specific tasks such as invasion, angiogenesis, immune surveillance and metastasis (Figure 1.4; Qian & Pollard 2010). Interestingly, TAMs have been found to populate pre-metastatic lesions producing a favourable environment for the establishment of metastases, also know as the 'pre-metastatic niche' (Joyce & Pollard 2009).

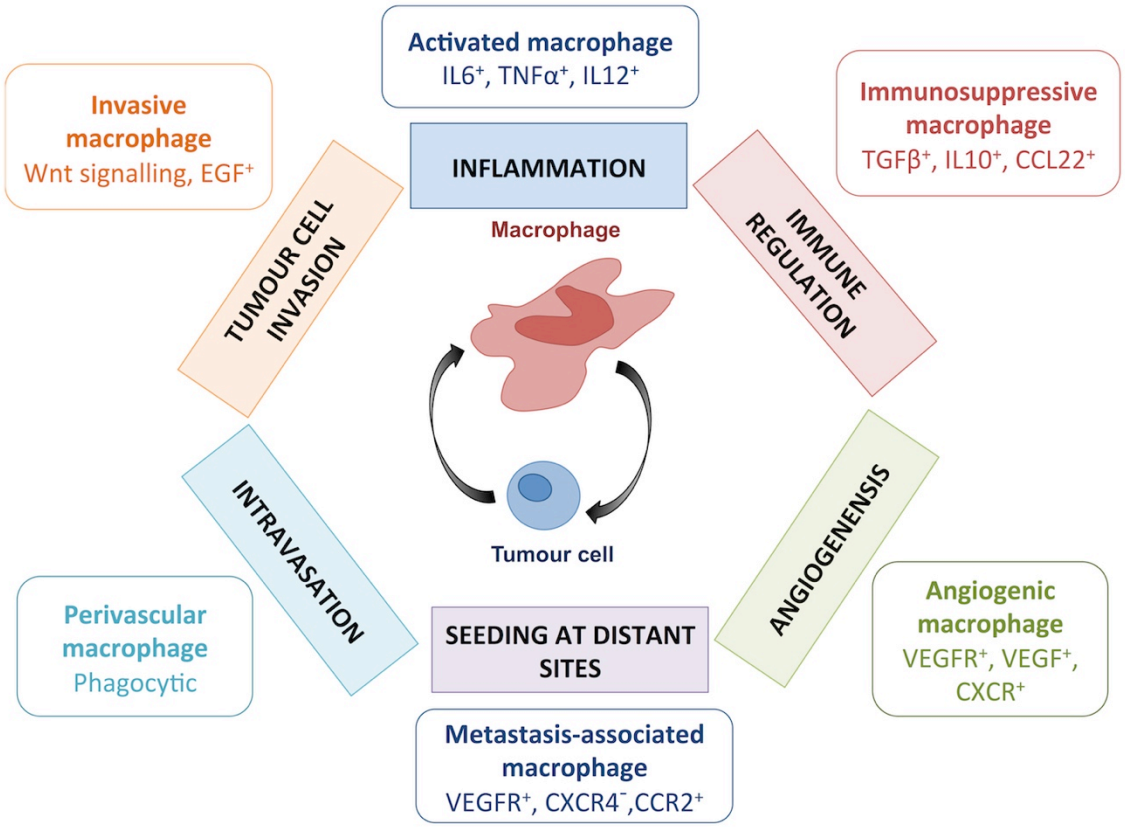

Figure 1.4 The macrophage population in the TME. Various different subtypes of macrophages exist in TME, each expressing a particular set of signals that have a well-defined role. These include activated macrophages (also known as M1), immunosuppressive or 'alternatively activated' macrophages (known as M2), angiogenic macrophages, metastatis-associated macrophages, perivascular macrophages and invasive macrophages. Adapted from Qian & Pollard 2010.

As with macrophages, neutrophils can be divided into anti-tumour (N1) and pro-tumour (N2) subpopulations. N1 cells generate a cytotoxic response against the tumour cells, either directly or by recruiting cytotoxic T cells to the tumour site, while N2 cells promoted by TGFβ are characterised by high expression of CCL2, VEGF and MMP9, which contribute to tumour progression (Sionov *et al.* 2014).

Macrophages and neutrophils at the tumour site recruit and activate various types of T cells. Cytotoxic T cells mediate tumour cell growth arrest and killing

by releasing interferon gamma (IFNγ) and inducing tumour cell lysis (Matsushita *et al.* 2015). In early stages of CRC, the presence of cytotoxic T cells in and around the tumour has been shown to be a predictor of patient survival (Reissfelder *et al.* 2015), suggesting that therapies promoting cytotoxic T cell activity could be effective for treating CRC. On the other hand, Tregs are responsible for suppressing pro-inflammatory immune responses by secreting anti-inflammatory cytokines such as IL10 and TGFβ to inhibit the activity of cytotoxic T cells (Facciabene *et al.* 2012). Th cells are another T cell type present at the tumour site: Th1 cells are necessary of the activation of cytotoxic T cells, while activated Th2 cells mediate a response similar to wound healing, which promotes tumour progression (Knutson & Disis 2005; Bhome *et al.* 2015). More importantly, the immune landscape appears to evolve with tumour progression. In CRC, the innate immune cells increase with tumour stage while the adaptive T cell population decreases (Bindea *et al.* 2013).

Therefore, it is apparent that the tumour fine-tunes its microenvironment to tip the balance towards pro-tumour signals by selectively manipulating the cell types and signals in its TME. An overview of the various cells types and signals in the TME is shown in Figure 1.5. Inflammatory signals including cytokine levels appear to be major contributors to this process.

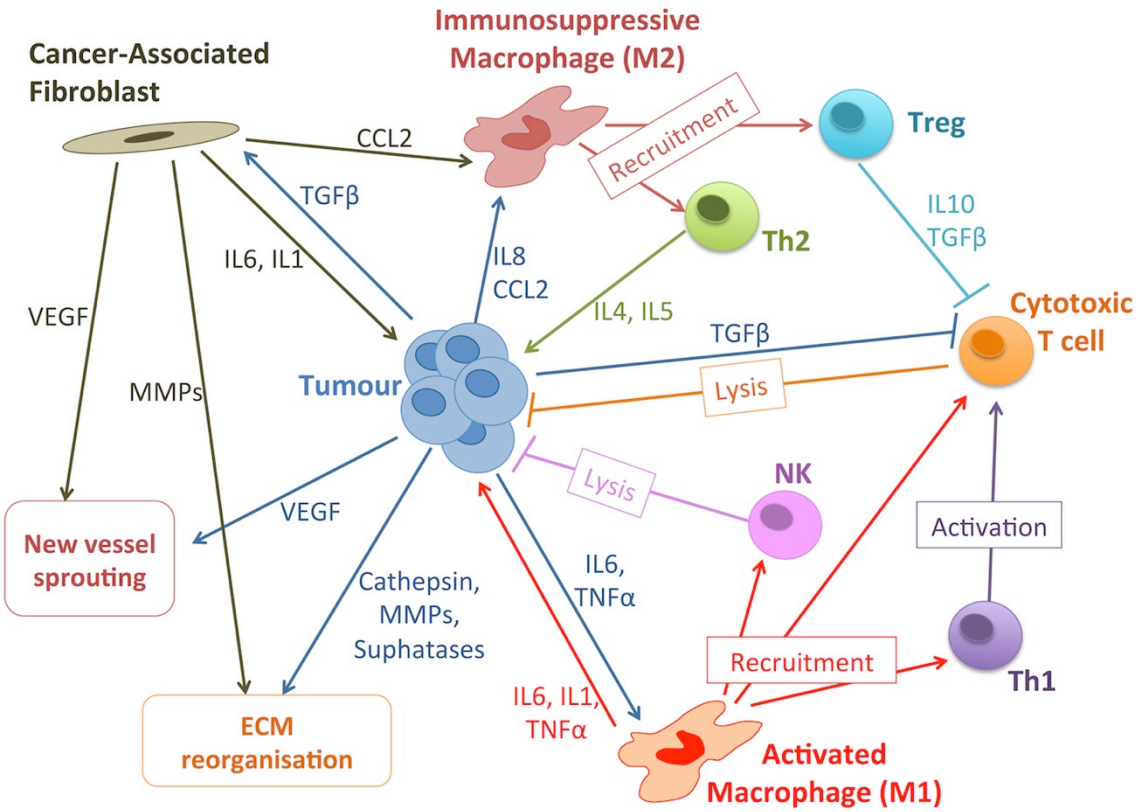

Figure 1.5 Overview of the TME. Tumour cells secrete a variety of signals resulting in ECM remodelling, angiogenesis, CAF differentiation, immune infiltration and suppression of anti-tumour immune response.

1.4 IL6, a key player in CRC?

Inflammation is part of the body's defence mechanism against infection and injury for 'self' preservation. Detection of 'non-self' leads to activation of inflammatory pathways resulting in recruitment of immune cells that destroy any 'non-self' and promote wound healing through tissue regeneration. While acute inflammation is therapeutic, chronic activation of inflammatory pathways is detrimental. Indeed, the inflammatory response is tightly controlled through cytokine-driven communication inducing pro-inflammatory signals to promote healing and anti-inflammatory signals when inflammation is no longer needed (Hanada & Yoshimura 2002). Deregulation of this response leads to chronic inflammation resulting in disease including autoimmune disorders, cardiovascular disease, arthritis, type 2 diabetes, IBD and cancer.

Over 150 years ago, Rudolf Virchow first hypothesised that inflammation plays a role in the development of cancer based on his observations that inflammatory cells infiltrate tumours (Balkwill & Mantovani 2001; Virchow 1863). In the last 30 years, extensive research on the topic has validated this idea and rekindled the interest of researchers in the cancer field. Chronic inflammation is now thought to promote various stages of carcinogenesis including cellular transformation, proliferation and survival, angiogenesis and metastasis (Aggarwal *et al.* 2006; Hu *et al.* 2015; Raposo *et al.* 2015; Kidane *et al.* 2014; Terzić *et al.* 2010). These effects are thought to be primarily mediated by inflammatory cytokines.

In the case of CRC, inflammation is thought to play an important role in both sporadic CRC and colitis-associated CRC (CAC). These two forms of CRC are thought to share many similarities and follow the same essential stages of CRC

development (Figure 1.2; Terzić *et al.* 2010). However, in CAC, chronic inflammation precedes cancer and thus contributes to CRC formation, while in sporadic CRC, the general view is that inflammation occurs following tumour formation. However, in the case of sporadic CRC, intestinal injury caused by external stimuli can also cause colonic inflammation (e.g. diet, refer to section 1.1). In both cases, inflammation in the colon induces ROS formation and oxidative damage to DNA (Westbrook *et al.* 2009) as well as promotes crypt regeneration (Pull *et al.* 2005), resulting in mutations leading to cancer. ROS can also inactivate DNA repair enzymes (Colotta *et al.* 2009), further promoting the frequency of mutations due to DNA replication errors. Furthermore, inflammation induced oxidative DNA damage has been shown to be gene-specific, affecting *p53* and *TGFβ receptor type II* genes, common mutations in CRC, with relatively no damage to the *β-Actin* gene (Choi *et al.* 2002). Interestingly, *p53* gene mutations have been shown to induce a pro-inflammatory microenvironment *via* NFκB activation in CRC tumours (Schwitalla *et al.* 2013). Hyper-activation of pro-inflammatory COX2 and NFκB pathways are commonly observed in CRC tissue (Charalambous *et al.* 2009; Terzić *et al.* 2010; Kraus & Arber 2009), which lead to aberrant cell growth through cytokine deregulation. In fact NFκB is activated in more than 50% of CRC tumours (Kojima *et al.* 2004; Karin & Greten 2005) and promotes expression of most pro-tumourigenic cytokines (Terzić *et al.* 2010; Marusawa & Jenkins 2014).

Pro-inflammatory cytokines are thought to be involved in cancer development and progression. These include tumour necrosis factor-α (TNFα), IL1β, IL8 and IL6. TNFα, IL8 and IL1β are thought to exhibit their tumourigenic effects

through activation of the NFκB pathway (Manna & Ramesh 2005; Karin 2009; Hoesel & Schmid 2013). The latter emerged as a promising target for cancer therapy, however further research revealed its inhibition could be cancer-promoting through suppression of p53 activity (Hellin *et al.* 1998) making it an unsuitable candidate. Therefore, in the search for a new therapeutic candidate IL6 signalling emerged as an attractive target as it has been shown to play a central role in mediating chronic inflammation and cancer (Scheller *et al.* 2006; Waldner *et al.* 2012; Chang *et al.* 2014; Taniguchi & Karin 2014; Terzić *et al.* 2010). IL6 is downstream of TNFα, IL8 and IL1β signalling, suggesting that these other pro-inflammatory cytokines may display their cancer-promoting effects in part through inducing IL6 expression.

IL6 is a pleiotropic cytokine with a wide range of biological effects including inflammation, immune regulation, haematopoiesis and oncogenesis. IL6 signals through a classical pathway where it binds to the IL6 receptor (IL6R) with low affinity on the cell surface (O'Reilly *et al.* 2013). IL6/IL6R complexes bind to the gp130 transmembrane receptor. The IL6/IL6R/gp130 complex then homodimerizes to form a hexameric complex (Murakami *et al.* 1993), which activates the Janus tyrosine kinase (JAK) family that in turn activates the signal transducers and activators of transcription 3 (STAT3) through phosphorylation. Activated STAT3 can then dimerize and translocate to the nucleus, where it can bind to the promoter regions of its target genes and induce transcription (Figure 1.4; O'Reilly *et al.* 2013; Jarnicki *et al.* 2010). IL6 can also signal through a non-classical pathway, in a process known as *trans*-signalling *via* the soluble IL6R (sIL6R), thus allowing IL6 to signal to cells lacking cell surface IL6R expression (Figure 1.4; O'Reilly *et al.* 2013). Under normal conditions, IL6/JAK/STAT3

pathway is rapidly terminated by the suppressor of cytokine signalling 3 (SOCS3) feedback inhibitor (Figure 1.4; Rigby *et al.* 2007). However, in chronic inflammation, inactivation of SOCS3 by proteolytic degradation or promoter hypermethylation results in aberrant activation of this pathway (Kim *et al.* 2015; Li *et al.* 2012). In addition to STAT3 signalling, IL6 can also activate other tumour-promoting pathways including ERK, MAPK and PI3K/AKT (Figure 1.6; Guo, Xu, Lu, Duan, & Zhang, 2012).

IL6 also seems to be particularly important in IBD and CRC pathogenesis (Scheller *et al.* 2006; Terzić *et al.* 2010; Ullman & Itzkowitz 2011; Waldner *et al.* 2012; Taniguchi & Karin 2014). IL6 *trans*-signalling in IBD is thought to promote chronic activation of effector T cells and resistance to apoptosis as well as inhibit differentiation of regulatory T cells, resulting in chronic activation of IL6 producing T cells in colonic tissue (Atreya *et al.* 2000). Furthermore, IL6 is associated with CRC tumour stage, size, metastasis and patient survival. IL6 is overexpressed at the tumour site in CRC patients (Maihöfner *et al.* 2003; Nagasaki *et al.* 2014; Chung *et al.* 2006; Lu *et al.* 2015; Uchiyama *et al.* 2012) and plasma levels of the cytokine correlate with tumour size and patient prognosis (Chung & Chang 2003; Uchiyama *et al.* 2012). Epidemiological studies have found that a gain of function polymorphism (-174 G>C) in the *IL6* gene promoter is associated with increased CRC risk (Landi *et al.* 2003; Kim *et al.* 2009; Woo & Humphries 2013). *In vitro*, IL6 was found to directly stimulate invasiveness (Hsu & Chung 2006) and proliferation of CRC cell lines with addition of anti-IL6 antibody resulting in inhibition of this effect (Schneider *et al.* 2000). Christian Becker and co-workers found that tumour growth *in vivo* using a mouse model for CRC was mediated by IL6 *trans*-signalling and

blockade of sIL6R or gp130 was sufficient to inhibit tumour growth (Becker *et al.* 2005). Tumour-associated immune cells have also been shown to secrete increased amounts of IL6 and sIL6R into the TME, thus promoting cancer progression through stimulation of IL6 *trans*-signalling in adjacent tumour cells (Matsumoto *et al.* 2010).

IL6 can also promote angiogenesis through direct vessel sprouting (Gopinathan *et al.* 2015) as well as through STAT3-mediated induction of VEGF expression (Waldner *et al.* 2010). STAT3 activation by IL6 is thought to mediate tumourigenesis in the colon by inducing expression of a number of genes that protect from apoptosis and promote cell cycle progression (*Bcl-2*, *Survivin*, *Hsp70*, *Cyclin D1*, *cMyc*; Bromberg & Wang, 2009) and inhibition of JAK2/STAT3 signalling induces CRC cell apoptosis (Du *et al.* 2012). In addition, SOCS3 disruption *in vivo* in a mouse model for sporadic CRC increased STAT3 signalling inducing crypt proliferation and resulting in increased colonic tumour load and size (Rigby *et al.* 2007). More recently, it was reported that IL6-mediated STAT3 activation in CRC cells prevented nuclear translocation of hMSH3, an important effector of DNA mismatch repair, thus promoting DNA damage and carcinogenesis (Tseng-Rogenski *et al.* 2014). In addition to STAT3 signalling, IL6 may also been shown to alter gene expression through epigenetic mechanisms including genome-wide methylation *via* induction of DNA methyltransferase 1 (DNMT1; Hodge *et al.* 2007; Hodge *et al.* 2005; Foran *et al.* 2010) and deregulation of microRNA (miRNA) expression such as miR21 (Löffler *et al.* 2007). MiRNAs are small RNA molecules involved in regulating most cellular pathways, and changes in their expression have been implicated in cancer; miRNAs could thus be important regulators of IL6-

mediated CRC and this is discussed in the following segment of this introduction (section 1.3).

IL6 therefore appears to play a key role in CRC development and progression, primarily *via* activation of STAT3 signalling. While STAT3 has emerged as a promising target for cancer therapy, targeting STAT3 signalling has proved quite challenging and numerous drugs that have been developed thus far do not have the desired potency (Furqan *et al.* 2013). Further understanding the mechanisms controlling IL6-mediated CRC carcinogenesis could lead to the discovery of new potential therapeutic strategies.

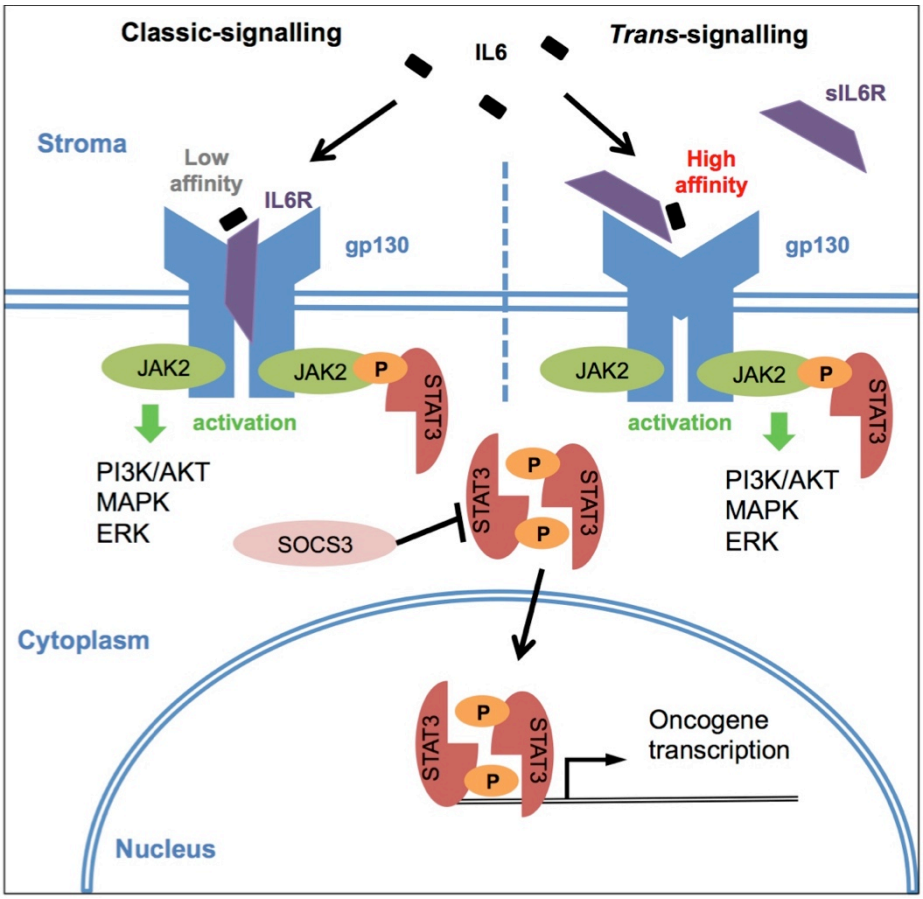

Figure 1.6 IL6 classic and *trans*-signalling pathways. In the classic pathway, IL6 binds to the IL6R membrane bound receptor with low affinity and signals through gp130 to activate JAK/STAT3 signalling. In *trans*-signalling, IL6 binds to the soluble IL6R (sIL6R) with high affinity, which interacts with gp130 and activates JAK/STAT3 signalling; this pathway is activated in chronic inflammation.

1.5 MiRNAs, master regulators of carcinogenesis?

While the Vogelstein model for colorectal carcinogenesis includes stepwise genetic mutations responsible for CRC initiation and progression, it was established at a time when non-coding DNA sequences were regarded as 'junk' with no particular function ascribed and the concept of epigenetics was not yet well understood. It is now known that factors other than DNA sequence are involved in controlling gene expression and phenotypic change. The study of these factors is referred to as epigenetics. Over the years, the Vogelstein model of CRC carcinogenesis has been modified to include epigenetic changes that contribute to carcinogenesis (Slaby *et al.* 2009).

Three main epigenetic mechanisms are known to regulate gene expression within cells: DNA methylation, histone modification and RNA silencing (Gibney & Nolan 2010). These processes play a crucial role in disease and particularly cancer. Regulation of gene expression by DNA methylation occurs *via* covalent addition of a methyl (CH_3) group to position C-5 on cytosine DNA residues located in specific gene promoter regions named CpG islands (GC-rich regions of 1000bp in length) resulting in gene silencing. Changes in DNA methylation in cancer were first discovered in 1983 (Feinberg & Vogelstein 1983). While oncogene promoter regions such as *ras* are hypomethylated (Feinberg & Vogelstein 1983), tumour suppressor genes such as *p53* are known to be silenced through hypermethylation. Histone modifications such as deacetylation are responsible for regulating chromatin condensation, which in turn controls gene expression (Gibney & Nolan 2010). Enzymes that catalyse these reactions are known as histone deacetylases (HDAC) and are overexpressed in human cancers. In CRC, where they have been linked to early

tumour initiating events such as loss of *APC* (Zhu *et al.* 2004) as well as later events such as metastasis (Choi *et al.* 2001). As for RNA silencing, non-coding RNA sequences transcribed from what was previously regarded as 'junk' DNA play a key role in regulating gene expression. At present four classes of regulatory non-coding RNAs are known: long non-coding RNAs, piwi-interacting RNAs, short interfering RNAs and miRNAs. While a full review of these various epigenetic mechanisms and their role in cancer are beyond the scope of this thesis, there are several excellent general reviews on these topics (Ropero & Esteller 2007; Johnstone 2002; Moazed 2009; Kaikkonen *et al.* 2011; Feinberg *et al.* 2002). For the purposes of this review, I will concentrate on describing miRNAs, their role in CRC and their interaction with IL6.

The study of miRNAs has gained a lot of momentum in recent years as emerging evidence shows these are involved in regulating most key cellular processes including differentiation, proliferation and apoptosis (He & Hannon 2004). The existence of miRNAs was only identified two decades ago with the discovery of lin4 in *C. elegans*, which is involved in larvae development and found to regulate *lin14* mRNA translation through binding the 3' untranslated region (UTR). This suggested a novel mechanism for regulating gene expression (Lee *et al.* 1993), however the implications of this discovery in humans and other animals was not understood at that time. It was only seven years later that a second miRNA, let7 was discovered (Reinhart *et al.* 2000). Unlike lin4, let7 had homologues in numerous species including human (Pasquinelli *et al.* 2000). It is now established that most miRNAs are highly conserved and expressed in most complex eukaryotes. New miRNAs are continuously being discovered and the most recent release of the miRNA database contains 35828 mature miRNAs

in 223 species with 2588 in humans alone (the June 2014 release 21 of the miRBase miRNA database; Griffiths-Jones 2004).

MiRNAs are transcribed from non-coding regions of the genome; these can either be intergenic regions or non-coding regions within genes also known as introns (He & Hannon 2004). They are on average 20-25 nucleotides in length. Their biogenesis pathway (Figure 1.7) begins with transcription of a primary miRNA (pri-miRNA) by RNA polymerase II or III, which is then folded onto itself and cleaved by Drosha RNA endonuclease to form stable RNA hairpin structures known as precursor miRNAs (pre-miRNAs, 60-80 bp in length). The latter are then transported from the nucleus to the cytoplasm by Exportin 5 and Ran-GTP where they are cleaved by Dicer to form miRNA duplexes (20-25 bp in length). One strand of the duplex (the mature miRNA) is selectively loaded onto the RNA-induced silencing complex (RISC), which identifies target mRNAs based on partial base complementarity resulting in mRNA cleavage or translational repression (He & Hannon 2004). Following this process, the miRNA remains intact and can proceed to bind to another target mRNA; thus one miRNA strand can affect multiple mRNA strands (He & Hannon 2004) suggesting that only small changes in miRNA expression are sufficient to significantly alter target expression. While miRNAs are generally thought to bind to the 3'UTR of target mRNAs to suppress expression, more recently a 5'UTR binding mechanism has been described (Kloosterman *et al.* 2004). Due to miRNAs only requiring partial base complementarity with their target sequences, it is thought that a single miRNA can target up to 200 different mRNAs (Krek *et al.* 2005) and 60% of mRNAs have complementary miRNA binding sites (Friedman *et al.* 2009).

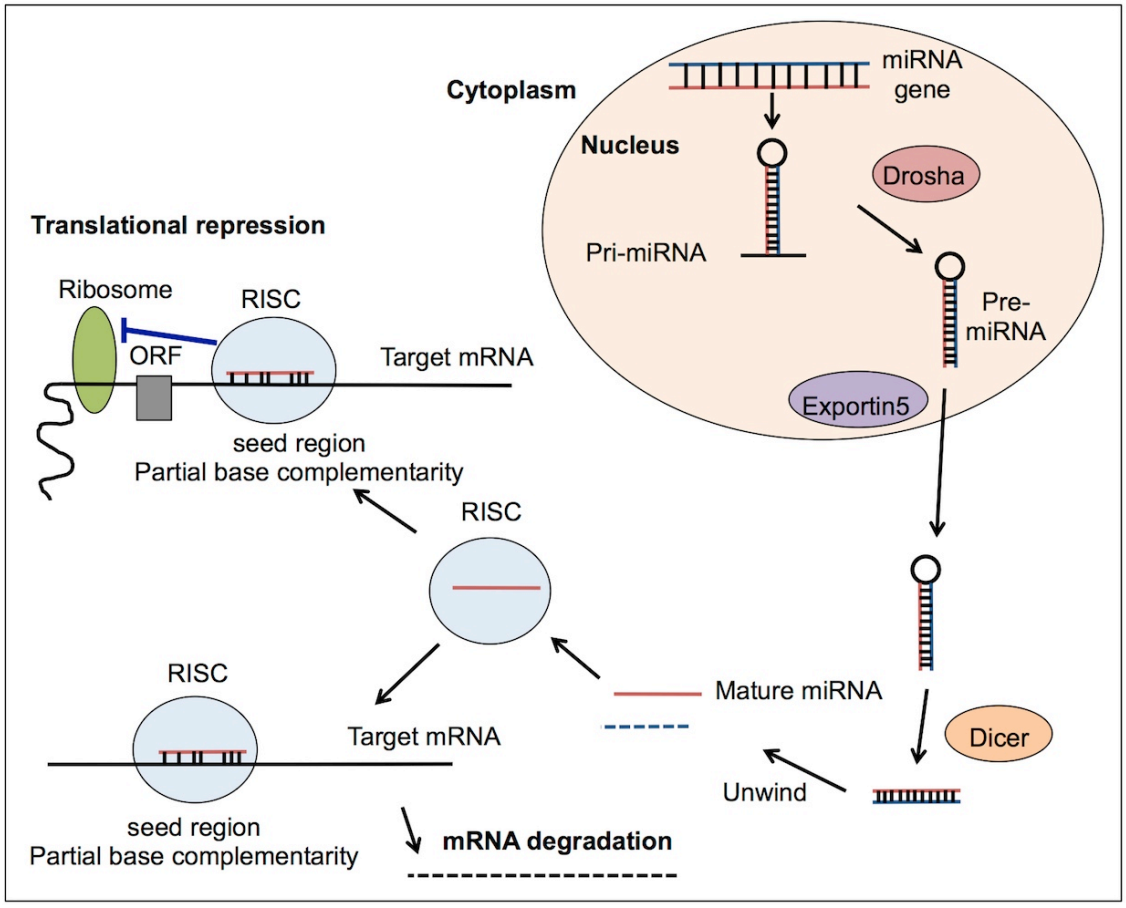

Figure 1.7 MiRNA biogenesis pathway. The miRNA gene is transcribed to form a pri-miRNA, which is cleaved by Drosha RNA endonuclease into the pre-miRNA. The latter is exported from the nucleus by Exportin5 into the cytoplasm, where it is cleaved by Dicer RNA endonuclease and unwound to yield the mature miRNA strand. The mature miRNA then associates with the RISC complex and binds to the mRNA seed region with partial base complementarity and induces either translational repression or mRNA degradation. Adapted from He & Hannon 2004.

MiRNAs have tissue-specific expression as they are involved in differentiation and specialisation of these tissues, thus their deregulation has significant consequences. Changes in miRNA expression in cancer cells was initially identified in B-cell chronic lymphocytic leukaemia (Calin *et al.* 2002) and it is now estimated that more than 50% of miRNA sequences occur in regions associated with chromosomal abnormalities in cancer (Calin & Croce 2006). Over the last decade, a breadth of research into miRNA expression and cancer

has led to the notion that aberrant expression of miRNAs plays an important role in carcinogenesis. Each malignancy has a unique miRNA profile including CRC (Table 1), thus miRNAs can differentiate human cancers and potentially act as biomarkers for disease (Calin & Croce 2006). MiRNAs with altered expression in cancer are either regarded as oncogenic or tumour-suppressive depending on the genes they regulate (Calin & Croce 2006).

The first miRNAs found to be downregulated in CRC, miR143 and miR145, were identified by high throughput profiling just over a decade ago. They are clustered on chromosome 5 around a fragile genomic region often deleted in cancer (Michael *et al.* 2003). Expression of these tumour-suppressive miRNAs is able to inhibit cell growth and invasion by inhibiting *cMyc* and *Mucin1* gene expression (Cui *et al.* 2014). Additionally, miR143 has been shown to target *KRAS*, an important oncogene in CRC carcinogenesis (Chen *et al.* 2009). Subsequent studies have identified many other differentially regulated miRNAs in CRC (Table 1; Pucci & Mazzarelli 2011, Yang *et al.* 2009, Nishida *et al.* 2011.), and selected examples involved in regulating key pathways described in the Vogelstein model for CRC carcinogenesis will be described here (Figure 1.8). MiR21 is upregulated in CRC and IBD; it targets pro-apoptotic and tumour suppressor genes including *PTEN* and has been associated with CRC mestastasis and poor prognosis (Asangani *et al.* 2008). MiR125b upregulation has also been associated with poor prognosis in CRC and has been shown to directly target *p53* mRNA (Nishida *et al.* 2011; Le *et al.* 2009). The miR200 family targets *ZEB1*, a key mediator EMT involved in tumour metastases (Brabletz *et al.* 2011), is transcriptionally regulated by p53 (Feng *et al.* 2011) and is downregulated in CRC (Yang *et al.* 2009). P53 is also thought to regulate

miR34a (Feng *et al.* 2011), another tumour-suppressor miRNA downregulated in CRC known to promote cell cycle arrest. Recently, a miR34 mimic became the first miRNA-based cancer therapy to reach phase I clinical trials (Agostini & Knight 2014). Loss of APC, a crucial step in early CRC carcinogenesis, may be mediated by upregulation of miR135b (Valeri *et al.* 2013). MiRNAs identified as being deregulated in CRC are thus crucial modulators of the cellular phenotype. Alterations in their expression appear to drive the process of carcinogenesis in the colon rather than be a mere consequence of it. However, the mechanisms regulating miRNA expression in cancer are not yet fully understood.

Interestingly, miRNAs deregulated in CRC are able modulate IL6 signalling. MiR34a can target *IL6R* and IL6-mediated activation of STAT3 represses miR34a expression (Rokavec *et al.* 2014). MiR124a and miR375 (downregulated in CRC) can target STAT3 and JAK2 mRNAs respectively (Koukos *et al.* 2013; Xu *et al.* 2014). IL6 mRNA is also a validated target of let7*,* which is also downregulated in CRC (Iliopoulos *et al.* 2009). IL6 is a known inducer of a number of tumour-promoting transcription factors with the potential to induce expression of different miRNAs, however only few miRNAs have been identified to be regulated by IL6, these include miR21 (as mentioned previously), miR370, miR148 and miR152 (Löffler *et al.* 2007; McCoy 2011). Therefore, miRNAs may underlie the strong link between IL6 and CRC and more importantly, IL6 may regulate key miRNAs involved in CRC progression.

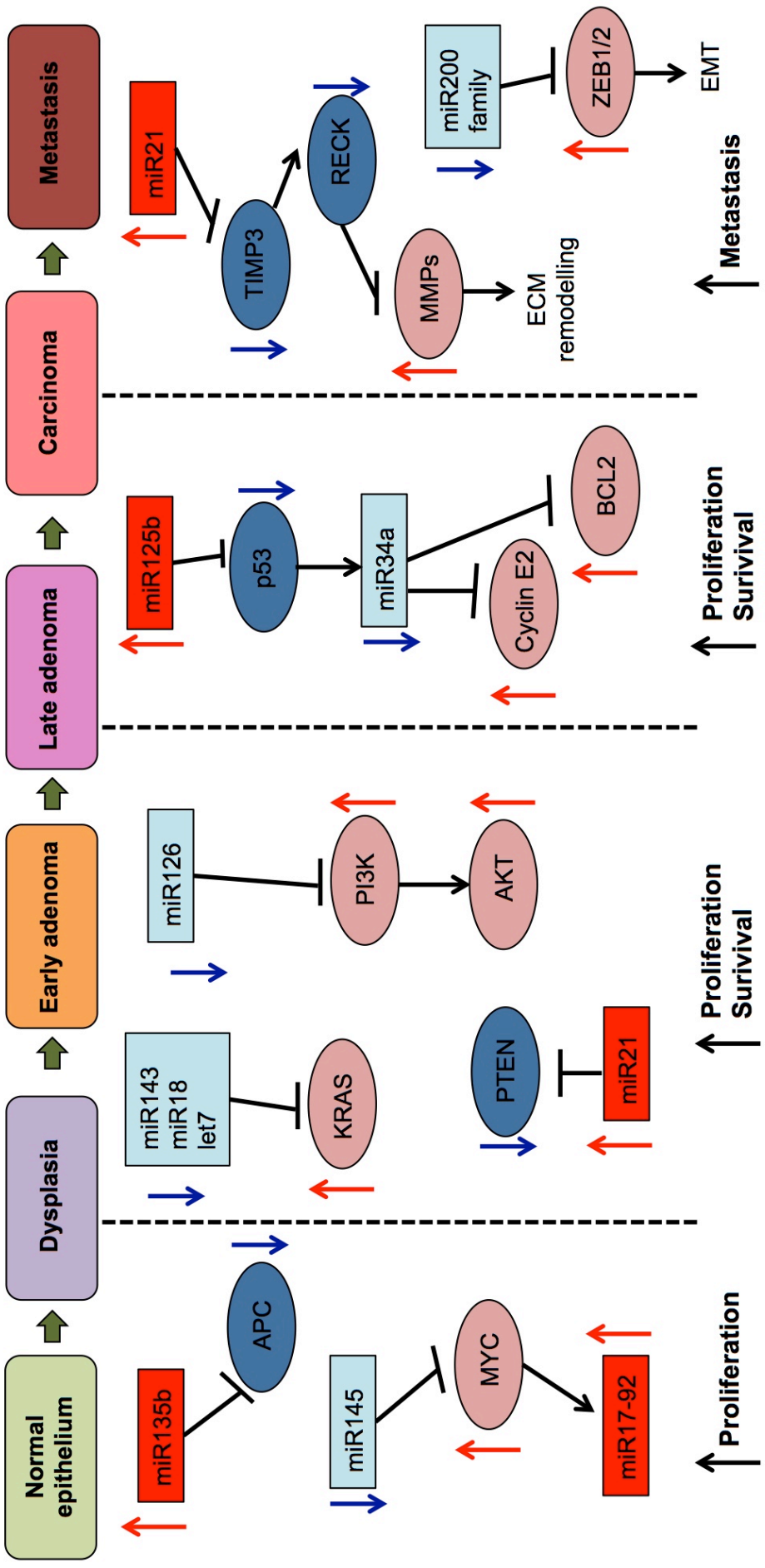

Figure 1.8 MiRNAs are involved in Vogelstein's model for CRC initiation and progression. Adapted from Slaby, Svoboda, Michalek, & Vyzula 2009.

Table 1. MiRNAs commonly deregulated in colorectal cancer. Adapted from Pucci & Mazzarelli 2011, Yang *et al.* 2009, Nishida *et al.* 2011.

Downregulated	Upregulated	Hypermethylated
let7a	miR106b-93-25	miR124
miR192	miR155	miR34b
miR34a	miR21	miR137
miR143	miR181b-1	
miR145	miR17-92 cluster	
miR215	miR96	
miR200 family	miR135b	
miR27b	miR125b	
miR124a		

1.6 Thesis hypothesis and objectives

Hypothesis

CRC development and progression appears to be strongly linked to lifestyle mainly through diet. Although it is speculated that dietary carcinogens are the main cause, mechanisms of their activation in the colon remain unclear. It is also well established that inflammation plays a key role in CRC and that IL6 appears to be essential to this process. Furthermore, in recent years epigenetic mechanisms and miRNAs in particular have emerged as important regulators of CRC progression, however the processes that regulate their expression in cancer are not yet well established. Determining these various mechanisms would bridge the gaps in the current understanding of CRC development and progression, and could possibly lead to the identification of better preventive strategies as well as therapeutic targets.

I hypothesise that elevated levels of IL6 in the CRC TME stimulate epigenetic and genetic alterations by inducing miRNA deregulation and local activation of dietary mutagens, thereby promoting CRC progression.

Objectives of project

I aim to investigate my hypothesis through the following objectives:

1. Investigate the role of IL6 on dietary pro-carcinogen-mediated DNA damage *in vitro* and determine whether miRNAs are involved in observed effects;
2. Determine the effect of IL6 treatment on epithelial CRC cell lines *in vitro* and investigate the underlying molecular mechanisms involved in phenotypic changes including selective mRNA and miRNA expression;

3. Interrogate changes in miRNA expression in response to IL6 in cultured CRC cells and investigate their role in IL6-mediated effects;
4. Investigate the role of IL6 in the TME using i*n vitro* co-culture of immune cells with CRC cells.

- Chapter 2 -

Interleukin-6 promotes dietary carcinogen-induced DNA damage by regulating cytochrome P450 enzymes.

N.B. Results from this chapter have been included in the following peer-reviewed publication:

Patel, S.A.A. and Gooderham, N.J., 2015. Interleukin-6 promotes dietary carcinogen-induced DNA damage in colorectal cancer cells. Toxicology Research, 4, pp.858-66 (Appendix D).

Patel, S.A.A. et al., 2014. Interleukin-6 mediated upregulation of CYP1B1 and CYP2E1 in colorectal cancer involves DNA methylation, miR27b and STAT3. British journal of cancer, 111(12), pp.2287-96 (Appendix D).

2.1 Introduction

CRC is largely regarded as an environmental disease and results from an accumulation of environment-induced genetic and epigenetic changes. Epidemiological studies have suggested a strong link between diet and colorectal carcinogenesis in part due to the presence of dietary pro-carcinogens such as BaP and PhIP (Chao *et al.* 2005). These pro-carcinogens require metabolic activation to their respective DNA-damaging agents by CYP450 enzymes. CYP2E1, CYP1B1 and CYP1A1 are found extra-hepatically and are overexpressed in CRC tissue (Patel *et al.* 2014; McFadyen *et al.* 2004; Murray *et al.* 1997), suggesting a potential for *in situ* metabolism and increased susceptibility to dietary carcinogen-induced mutations. Therefore, CYP450s may play a crucial role in diet-induced CRC.

Pro-inflammatory cytokine IL6 is thought to play a central role in carcinogenesis and overexpression of IL6 occurs at the tumour site of multiple cancer types including CRC (Maihöfner *et al.* 2003; Chung *et al.* 2006; Lu *et al.* 2015; Uchiyama *et al.* 2012). In addition, diets with a high animal fat content can promote local inflammation in the colon (Erdelyi *et al.* 2009), suggesting that diet may promote CRC through induction of IL6, however the underlying mechanisms remain unknown. IL6 is known to regulate CYP450 enzymes. Previous studies in hepatocytes have generally reported that IL6 inhibits CYP450 expression (Abdel-Razzak *et al.* 1993; Jover *et al.* 2002; Hakkola *et al.* 2003). However, conflicting reports exist in different tissue types (Smerdová *et al.* 2014; Tindberg *et al.* 1996), suggesting that the effect of IL6 on CYP450 expression may vary depending on tissue type and the relation between

overexpression of IL6 and CYP450 expression in CRC has not been widely investigated.

In vitro techniques commonly use cells cultured as two-dimensional (2D) monolayers. However, when cultured in this manner, cells are flattened onto the culture surface thus changing their structure, and parameters such as cell-to-cell interaction and tissue architecture are lost (Figure 2.1). *In vitro* three-dimensional (3D) cell culture systems in which cells are grown as spheroids constitute a better model of *in vivo* tissue without the use of animal models. Previous studies comparing 2D and 3D cell culture systems have shown 3D cell morphology and growth rate more accurately represents *in vivo* tumours (Pampaloni *et al.* 2007). Thus, using 3D cell culture could increase the *in vivo* relevance of *in vitro* experimental results and here I have used this novel culture method to validate findings from 2D cell culture.

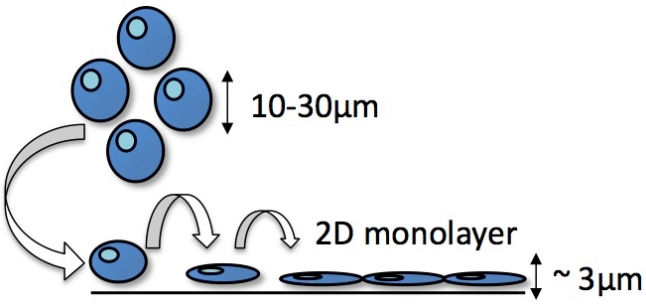

Figure 2.1 Classic cell culture method changes cell structure. Cells are flattened onto the plastic culture surface and cell-to-cell interaction is very limited.

In this chapter, I have investigated mechanisms involved in diet-associated CRC by studying the effect of IL6 on BaP- and PhIP-induced DNA damage as well as underlying mechanisms for observed effects using *in vitro* models. Exploring these mechanisms is important to further understand the role of IL6 in diet-associated colorectal carcinogenesis and could potentially lead to the identification of novel preventive or therapeutic strategies.

2.2 Materials and methods

2.2.1 Cell culture

The human colorectal adenocarcinoma cell lines HCT116 and SW480 (detailed in Table 2) were obtained from ATCC (LGC Prochem, Middlesex, UK). HCT116 p53-/- cells were kindly gifted by Professor Bert Vogelstein (John Hopkins University, Baltimore, MD, USA) and provided by Professor David Phillips (King's College London, UK). Cells were routinely cultured in RPMI1640 medium (GIBCO, Life technologies, Paisley, UK) supplemented with 10% foetal bovine serum (FBS), 100units/ml penicillin, 100μg/ml streptomycin and 2mM *L*-glutamine (GIBCO, Life technologies). All cells were incubated at 37°C in a humidified incubator (5% CO_2). Cells between passages 3-7 were used for experiments. For 3D cell culture, cells were seeded at a density of 5 x 10^5 cells/well in a 24-well Algimatrix system (Invitrogen, Life technologies) according to the manufacturer's protocol. Cells were monitored and culture medium was changed routinely. Spheroids cultured for 7-10 days were used in all experiments (Figure 2.2). To isolate spheroids from the matrix, matrix-dissolving buffer (Invitrogen, Life technologies) was used according to the manufacturer's protocol.

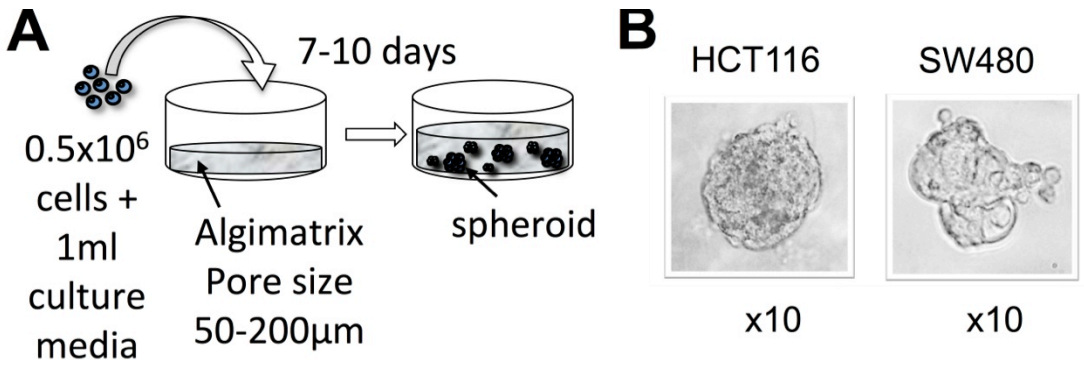

Figure 2.2 Three-dimensional cell culture. (A) 3D culture method using Algimatrix. (B) Pictures of HCT116 and SW480 spheroids (x10 magnification).

Table 2. Characteristics of HCT116 and SW480 cell lines.

Feature	HCT116	SW480
Organism	*Homo sapiens*	*Homo sapiens*
Tissue	Colon	Colon
Morphology	Epithelial	Epithelial
Disease	Colorectal adenocarcinoma	Colorectal adenocarcinoma
Stage	Dukes' type D	Dukes' type B
Gender	Male	Male
Doubling time	16 hours	20 hours
***KRAS* mutation**	Heterozygote mutant (GGC to GAC in codon 13)	Homozygote mutant (GGT to GTT mutation in codon 12)
p53 status	Wild-type	Mutant (G273A, C309T) constitutively activated
APC status	Wild-type (2843 residues)	C-terminus truncated at residue 1338
β-catenin status	Heterozygote mutant, stabilising deletion at S45 residue	Wild-type
Microsateillite stability	Instable	Stable
Repair pathway	Mismatch repair deficient	Mismatch repair proficient

2.2.2 Cell treatments

Prior to treatment, HCT116 and SW480 were maintained in culture medium supplemented with 5% dextran-coated charcoal-stripped FBS for at least 72 hours. Charcoal-stripping serum allows for the removal of hormones, growth factors and cytokines.

Cells were seeded at a density of $1x10^5$ cells per well of a 6-well plate (for 2D cell culture). IL6 was not expressed in HCT116 and SW480 cells (gene expression assays yielded unquantifiable signals, Figure S.4 in Appendix A). Human recombinant IL6 produced in HEK293 cells was obtained in the form of a lyophilised powder (HumanKine, Sigma-Aldrich, Dorset, UK) and rehydrated in PBS containing 0.1% human serum albumin (Sigma-Aldrich). IL6 was added to the cells for 24 and 48 hours at doses of 0-5000pg/ml (chosen within the range secreted by stromal cells in the colorectal TME; Nagasaki *et al.* 2014).

For treatment with dietary carcinogens, cells were pre-treated with IL6 as described above, washed with PBS and treated with a dose-range of BaP (0-10μM, Sigma-Aldrich) and PhIP (0-100μM, Toronto Research Chemicals Inc., Toronto, Canada). Both chemicals were dissolved in DMSO and final vehicle control concentration of 0.2% was used.

For demethylation and STAT3 inhibition, cells were co-treated for 24 and 48 hours with 1000pg/ml IL6 and either 4μM 5-aza-2'-deoxycytidine (Sigma-Aldrich) or 25μM STAT3 inhibitor (STAT3 inhibitor VIII 5,15-diphenylporphyrin, Millipore, Feltham, UK), respectively. STAT3 inhibitor and 5-aza-2'-deoxycytidine were dissolved in DMSO (vehicle control concentration of 0.1%).

2.2.3 Micronucleus assay

Micronucleus (MN) assay was performed according to OECD guidelines. Cells were seeded at a density of $5x10^4$ cells per well of a 24-well plate for 2D cell culture. Spheroids grown for 10 days were used for 3D culture. Cells were treated with IL6, BaP or PhIP as detailed in section 2.2.2, washed with PBS and cultured for a further 72 hours prior to harvest. Etoposide (Sigma-Aldrich) was used as a positive control in all assays at a concentration of 125nM as it is a known inducer of MN formation. Cells were then harvested and resuspended in culture medium containing 2% pluronic (GIBCO, Life technologies). Cell survival was determined by counting the number of viable cells using a haemocytometer with Trypan-Blue exclusion as per manufacturer's protocol (GIBCO, Life technologies). Cells were fixed with 100% methanol onto microscope slides at a density of $2x10^4$ cells per slide and stained for 60 seconds with acridine orange (0.1mg/ml dissolved in PBS, Sigma-Aldrich). Frequency of MN was scored blind in 1000 cells per sample and three biological replicates were performed per treatment (Figure 2.3).

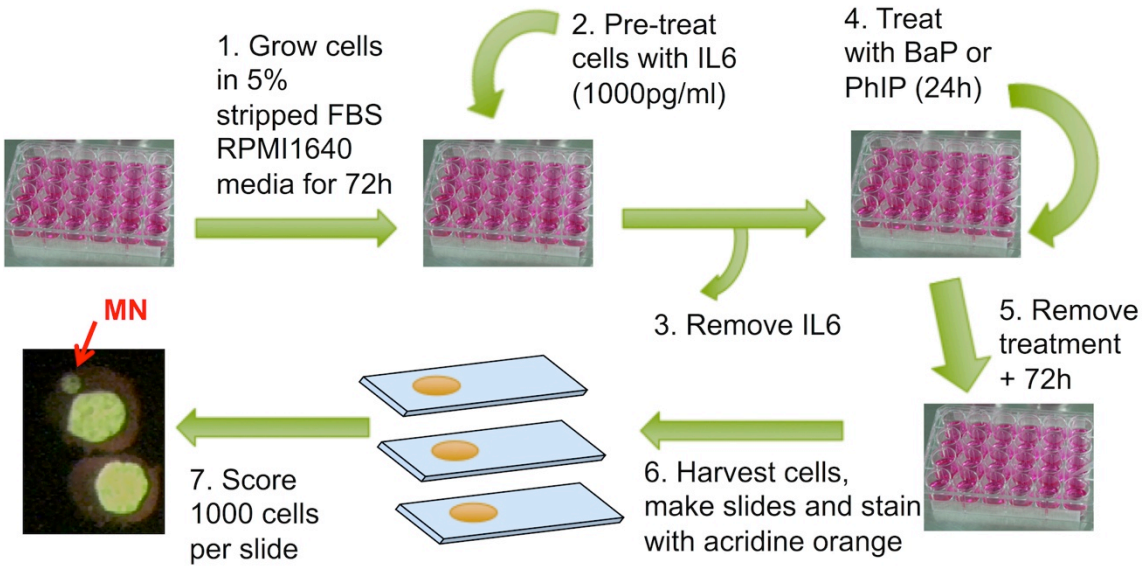

Figure 2.3 Micronucleus assay protocol.

2.2.4 RNA extraction

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Life technologies) according to the manufacturer's protocol. RNA extracts were quantified by UV spectroscopy (UV-VIS Nano-spectrophotometer, Implen, Essex, UK) with purity assessed from 260/280nm and 260/230nm ratios. Extracts were stored at -80°C until used.

2.2.5 Reverse transcription and quantitative-Polymerase Chain Reaction

For mRNA reverse transcription, RNA extracts (100-500ng) from each sample were added to 300ng of random primers, heated for 5min at 65°C and then immediately placed on ice. Each sample was incubated with 0.5mM dNTPs, 1x first strand buffer, 8μM dithiothreitol and 100units of Superscript II reverse transcriptase (Invitrogen, Life technologies) for 10min at 25°C, 90min at 42°C and 15min at 70°C on a thermocycler (Peltier Thermal Cycler PTC-200, MJ Research, Waltham, Massachusetts, USA). A miRNA reverse transcription kit was used for miRNA expression according to the manufacturer's protocol (Taqman, Applied Biosystems, Life technologies). Quantitative-Polymerase Chain Reaction (qPCR) was performed using pre-designed gene expression assays (detailed in Appendix A) and FAST PCR master mix (Taqman, Applied Biosystems, Life technologies), and measured in a StepOnePlus fast real-time PCR system (Applied Biosystems, Life technologies) according to the manufacturer's protocol. *GAPDH* and U6 expression were used to normalise cellular mRNA and miRNA expression respectively (Appendix A). Gene expression was quantified using the delta-C_t method.

2.2.6 Fluorescence detection of ethoxyresorufin-O-deethylase activity

CYP1A and CYP1B1 enzymes catalyse the O-deethylation of 7-ethoxyresorufin (7-ER) to fluorescent product resorufin, thus the ethoxyresorufin-O-deethylase (EROD) activity of CYP1A/1B1 enzymes can be measured by quantifying the amount of resorufin produced in the presence of 7-ER**.** Cells were seeded at a density of $1x10^5$ cells per well of a 24-well plate in 1ml of stripped medium and treated as previously described (section 2.2.2). Following treatment, cells were washed once with PBS and incubated with 8μM 7-ER for 4 hours at 37° C. Fluorescence measurements were taken at λ excitation=560nm and λ emission=590nm every 10 minutes using a fluorescence plate reader (Fluostar, BMG Labtech, Ortenberg, Germany). A resorufin standard curve was prepared over the range 0-5000pmol per 1ml of culture medium. Subsequently, total protein was extracted from cells collected in each assay and quantified as described in section 2.2.9. Activity rate was calculated as follows:

1. Blank measurement subtracted from all fluorescent measurements;
2. Fluorescence *vs* time (min) plotted and gradient g calculated from the linear portion of the graph;
3. Using resorufin standard curve, g converted into pmol resorufin/min;
4. Data set normalised to amount of protein contained within each sample;
5. Activity rate expressed as pmol resorufin/mg protein/min.

2.2.7 Transfection of miR27b inhibitor

MiR27b inhibitor was obtained from miRIDIAN (Thermo Fisher Scientific, Cramlington, UK) and transfected into the cells using Lipofectamine 2000

(Invitrogen, Life technologies) according to the manufacturer's protocol. Briefly, cells were seeded at a density of $1x10^5$ cells per well of a 24-well plate and cell culture medium was replaced by 400µl/well of Opti-MEM (GIBCO, Life technologies) prior to the addition 150µl/well of Opti-MEM containing 8µl of Lipofectamine 2000 reagent and 2.5µl of 20µM stock of miRNA inhibitor or miRIDIAN miRNA negative control. Cells were incubated with the transfection complexes for 6 hours prior to harvest.

2.2.8 Chromatin immunoprecipitation-qPCR

Four million SW480 cells were treated with 1000pg/ml IL6 and 25µM STAT3 inhibitor for 60min prior to harvest. Chromatin Immunoprecipitation (ChIP) assay was performed using a magnetic ChIP kit (Thermo Fisher Scientific) as per manufacturer's protocol with an anti-STAT3 antibody (sc-482X, Santa Cruz Biotechnology, Heidelberg, Germany). PCR primers were designed to amplify STAT3 binding sites of interest using Primer-BLAST (NCBI, Ye *et al.*, 2012). Rabbit IgG was used as a negative control for non-specific binding. Binding was calculated as a percentage of the total input chromatin. Sequences of the primers used were as follows:

CYP2E1 site 1	forward	5'-TGAATTTTCCTTCTGGCCCCAT-3',
	reverse	5'-TGATGAGGAGGTTTGTCTGAGC-3';
CYP2E1 site 2	forward	5'-CTCCATCCTCACCAGGTCAC-3',
	reverse	5'-CCAACCAATGCCCTCTTGCT-3'.

2.2.9 Immunoblotting

Cells were treated as described in section 2.2.2. Lysates were prepared from the treated cells using RIPA buffer (Sigma-Aldrich) as per manufacturer's

instructions and quantified by BCA assay (Thermo Fisher Scientific). Immunoblotting was performed as previously described (Lauber & Gooderham 2011) and 30μg of protein sample was loaded into each well of a 10% SDS-polyacrylamide gel. Anti-STAT3 (ab50761), anti-pSTAT3 (ab32143) and secondary antibodies (Abcam, Cambridge, UK) were used at a dilution of 1:10000. Blots were also incubated with anti-β-actin antibody (A2228, 1:10000 dilution, Sigma-Aldrich), which was used as an internal loading control. Target protein bands were visualised using Luminata Forte chemiluminescent reagent (Millipore). Blots were exposed and quantified using the Kodak image station 4000MM. (Kodak, Watford, UK).

2.2.10 Statistical analysis

Data were obtained from measurements made in at least three biological replicates and presented as a mean ± standard error (SEM). Significant differences ($p<0.05$) were determined using Student's *t*-test, one-way analysis of variance (ANOVA) followed by a Dunnett post-test or a linear trend analysis and two-way ANOVA followed by a Bonferroni post-test. Pearson's product-moment correlation coefficient test was used for correlation analysis (GraphPad Prism 5, GraphPad Software Inc., La Jolla, CA, USA).

2.3 Results

2.3.1 IL6 promotes chemical-induced DNA damage by food-derived pro-carcinogens BaP and PhIP.

The *in vitro* MN assay is a commonly used toxicological test for detecting genotoxic potential of compounds due to its simplicity of scoring, accuracy and adaptability to different cell types. More importantly, studies in humans have shown strong associations between micronuclei frequency and cancer risk (Bonassi *et al.* 2011). Formation of micronuclei, i.e. small membrane-bound DNA fragments in the cytoplasm, occurs during cell division when a whole chromosome or a chromosomal fragment is not incorporated into the nucleus of one of the daughter cells. Standard MN assays utilise immature blood cells, but non-standard versions of the assay have been developed using different cell types including epithelial cells. Here, I have adapted the assay for use with human colorectal adenocarcinoma epithelial cell lines grown as 2D and 3D cultures. I determined the optimum post-treatment time point for scoring the micronuclei in this system as 72 hours, since this was the time-point at which the highest number of MN were observed following treatment with BaP (Figure 2.4).

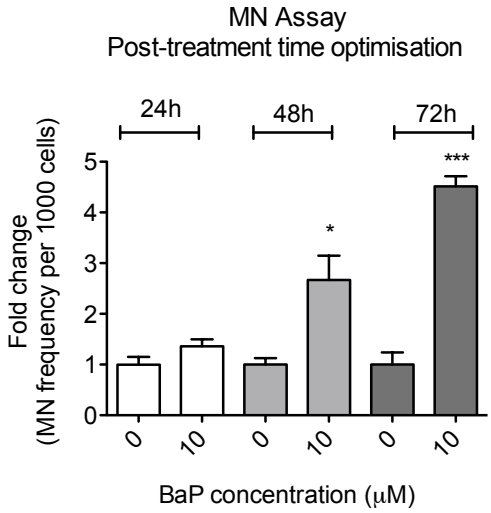

Figure 2.4 Micronucleus assay optimisation. HCT116 cells grown as monolayers were treated with 10μM BaP for 24 hours; cells were taken 24, 48 and 72 hours post-treatment and scored for micronuclei (MN) frequency. Data are expressed as fold change compared to vehicle control at each time point. Significance was assessed using Student's *t*-test comparing treated group to control (GraphPad Prism 5, *p<0.05, ***p< 0.001). Error bars represent the SEM for independent cultures (n=3).

Using the optimised method, a dose-dependent increase in BaP- and PhIP-induced MN frequency in HCT116 and SW480 cell lines cultured in 2D was observed. Interestingly, pre-treatment with IL6 significantly enhanced the pro-carcinogen-induced DNA damage while treatment with IL6 on its own had no effect (Figure 2.5 A, B and 2.6 A, B). In addition, HCT116 was more susceptible to DNA damage compared to SW480 cells possibly due to the lack of mismatch repair in HCT116 (Table 2). Cytotoxicity was also measured to ensure cell viability post-treatment. While some toxicity with high dose BaP and IL6 was observed, the differences were not statistically significant compared to control (Figure 2.5 C, D, 2.6 C, D).

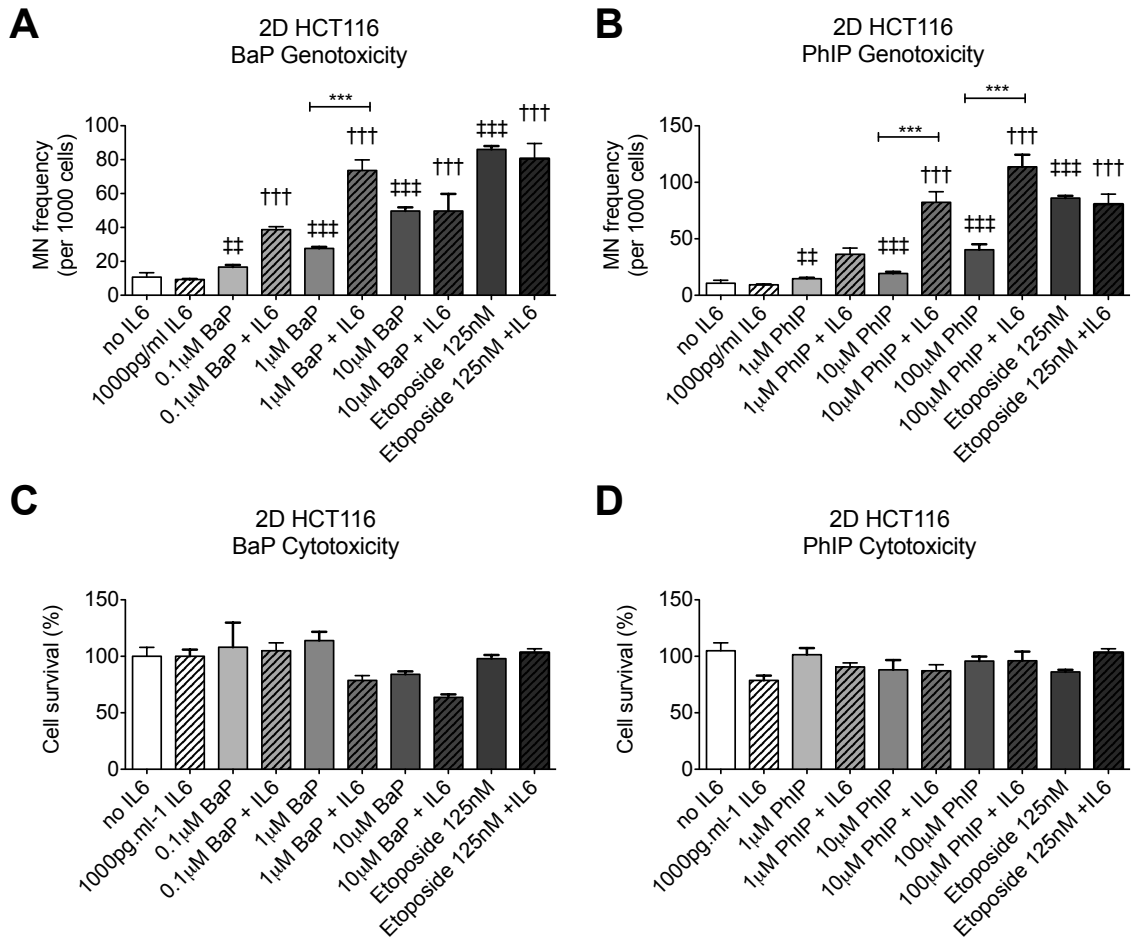

Figure 2.5 Micronucleus assay with 2D HCT116. HCT116 cells grown as monolayers were pre-treated with IL6 for 48 hours followed by a 24 hour treatment with BaP or PhIP; cells were taken 72 hours post-treatment. Etoposide was used as a positive control. (A, B) Micronuclei (MN) frequency per 1000 cells following treatment. (C, D) Cytotoxicity following treatment expressed as % of cell survival. Statistically significant differences are shown for comparisons between carcinogen treated *vs* IL6 pre-treated samples (*p<0.05, **p<0.01, ***p<0.001), IL6 alone *vs* IL6 pre-treated and carcinogen treated (†p<0.05, ††p<0.01, †††p<0.001) and vehicle *vs* carcinogen treated (‡p<0.05, ‡‡p<0.01, ‡‡‡p<0.001). Significance was calculated using one-way ANOVA with a Dunnett post-test (GraphPad Prism 5). Error bars represent the SEM for independent cultures (n=3). Adapted from Patel & Gooderham 2015.

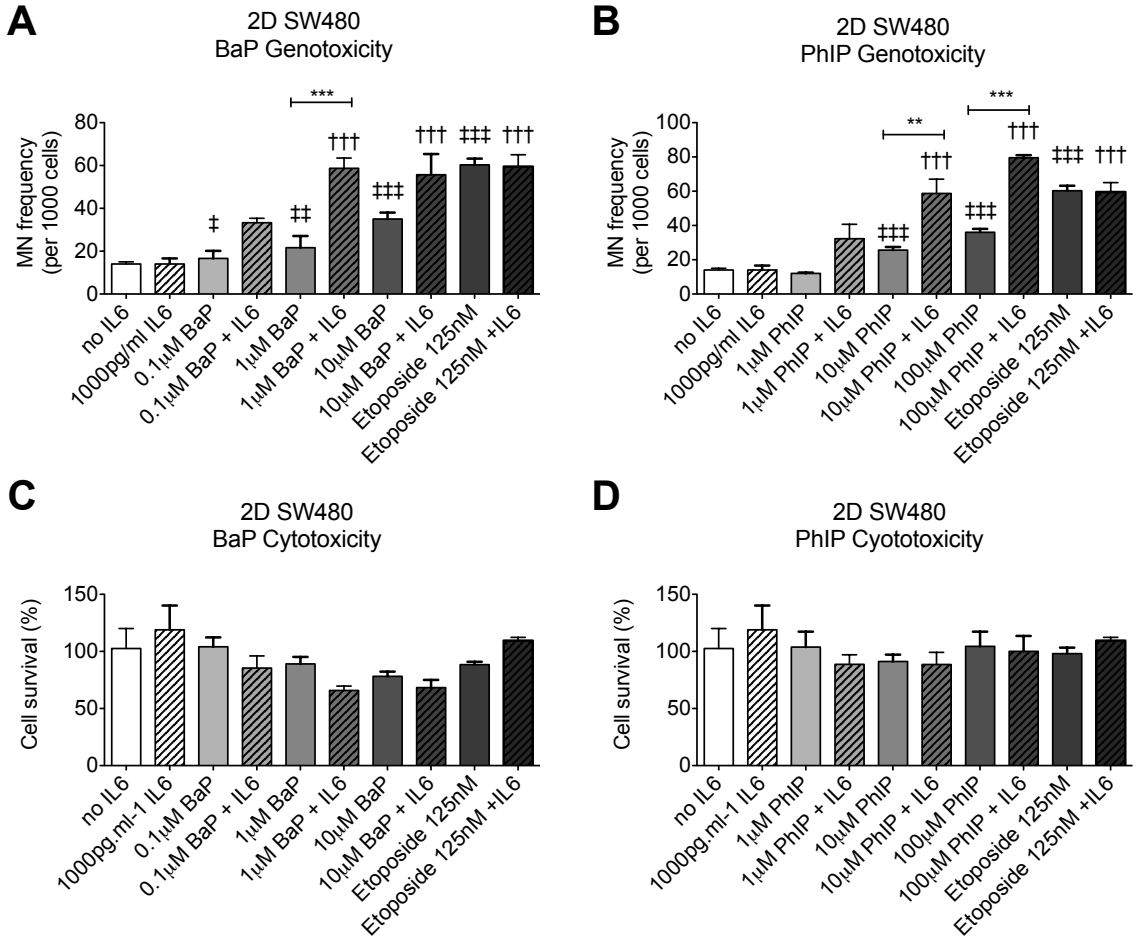

Figure 2.6 Micronucleus assay with 2D SW480. SW480 cells grown as monolayers were pre-treated with IL6 for 24 hours followed by a 24 hour treatment with BaP or PhIP; cells were taken 72 hours post-treatment. Etoposide was used as a positive control. (A, B) Micronuclei (MN) frequency per 1000 cells following treatment. (C, D) Cytotoxicity following treatment expressed as % of cell survival. Statistically significant differences are shown for comparisons between carcinogen treated *vs* IL6 pre-treated samples (*p<0.05, **p<0.01, ***p<0.001), IL6 alone *vs* IL6 pre-treated and carcinogen treated (†p<0.05, ††p<0.01, †††p<0.001) and vehicle *vs* carcinogen treated (‡p<0.05, ‡‡p<0.01, ‡‡‡p<0.001). Significance was calculated using one-way ANOVA with a Dunnett post-test (GraphPad Prism 5). Error bars represent the SEM for independent cultures (n=3). Adapted from Patel & Gooderham 2015.

The assay was repeated using HCT116 cells grown as 3D spheroids. In general, results were in agreement with that found in 2D culture (Figure 2.7). However, the increase in DNA damage observed with IL6 pre-treatment was not as pronounced in 3D culture compared to 2D. MN frequency was increased by 1.5 fold in 3D culture with IL6 pre-treatment, whereas in 2D culture, it was increased by 2-3 fold. Furthermore, MN frequencies with BaP and PhIP alone were increased in 3D cells compared to 2D cells. Etoposide, a topoisomerase II inhibitor and potent inducer of MN formation, was used as a positive control in all assays and does not require activation to induce DNA damage. Pre-treatment with IL6 did not enhance etoposide-induced DNA damage suggesting that IL6 may have an effect on the activation pathway of the pro-carcinogens rather than on induction of MN formation.

Therefore, these data demonstrate that presence of IL6 in colon epithelial cells along with food-derived pro-carcinogen can enhance DNA damage suggesting a potential role for IL6 in promoting cancer development and progression in the colon.

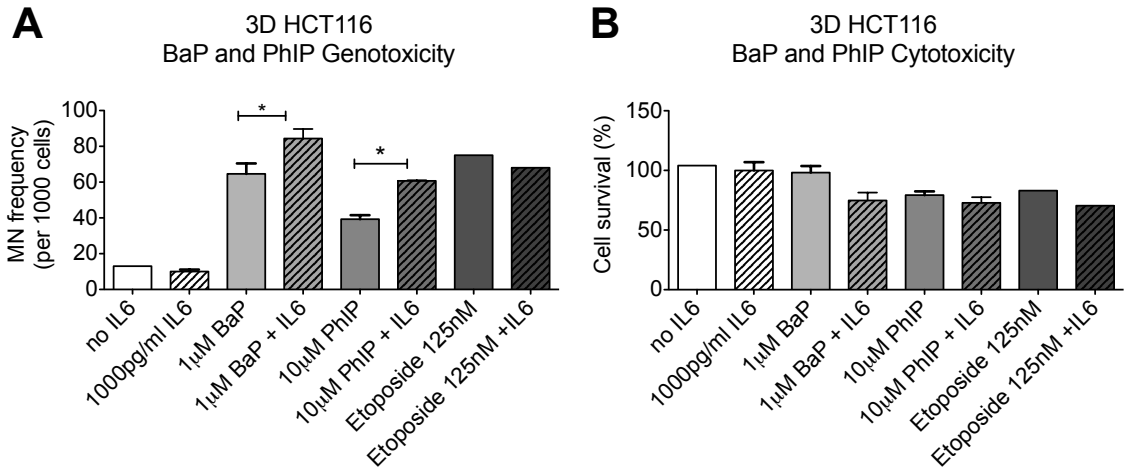

Figure 2.7 Micronucleus assay with HCT116 grown as 3D spheroids. Cells grown as 3D spheroids on Algimatrix were pre-treated with IL6 for 24 hours followed by a 24 hour treatment with BaP or PhIP; cells were taken 72 hours post-treatment. Etoposide was used as a positive control. (A) Micronuclei frequency per 1000 cells following treatment. (B) Cytotoxicity following treatment expressed as % of cell survival. Statistically significant differences are shown for comparisons between carcinogen treated *vs* IL6 pre-treated samples (*p<0.05, **p<0.01, ***p<0.001). Significance was calculated using one-way ANOVA with a Dunnett post-test (GraphPad Prism 5). Error bars represent the SEM for independent cultures (n=3). Reproduced from Patel & Gooderham 2015.

IL6 has been reported to downregulate *p53* gene expression (Hodge *et al.* 2005). The tumour suppressor protein p53 is crucial to maintain genomic stability and is commonly deactivated in cancer. Both of the cell lines used in this study have p53 activity. To test whether inactivation of p53 may be responsible for the IL6 effect observed, I performed a MN assay on HCT116 p53-/- cells obtained from Professor Bert Vogelstein's laboratory (John Hopkins University, Baltimore, MD, USA) and as well, I looked at the effect of IL6 treatment on *p53* gene expression by qPCR. No significant downregulation of *p53* gene expression was observed at the dose of IL6 used in this study (Figure 2.8 A, B and C). In addition, MN frequency was not increased in the p53-null

HCT116 cells when compared to wild-type HCT116, rather total number of MN was decreased (Figure 2.6 A and B, Figure 2.8 E). Therefore, this demonstrates that the observed effect of IL6 on MN induction by BaP and PhIP is not caused by downregulation of *p53*, further suggesting that IL6 may affect the activation pathway of the pro-carcinogens.

Previous studies in this laboratory have demonstrated that overexpression of IL6, CYP1B1 and CYP2E1 occurs in malignant tissue resected from CRC patients (Patel *et al.* 2014), indicating that IL6 may be associated with a change in metabolic competency. BaP and PhIP are both activated to their genotoxic form intracellularly by CYP1A1 and CYP1B1 enzymes. In addition, CYP2E1 is also known to activate dietary carcinogens such as acrylamide, found in starchy foods cooked at high temperatures. Therefore, I proceeded to investigate the effect of IL6 on *CYP1A1*, *CYP1B1* and *CYP2E1* expression in CRC cells.

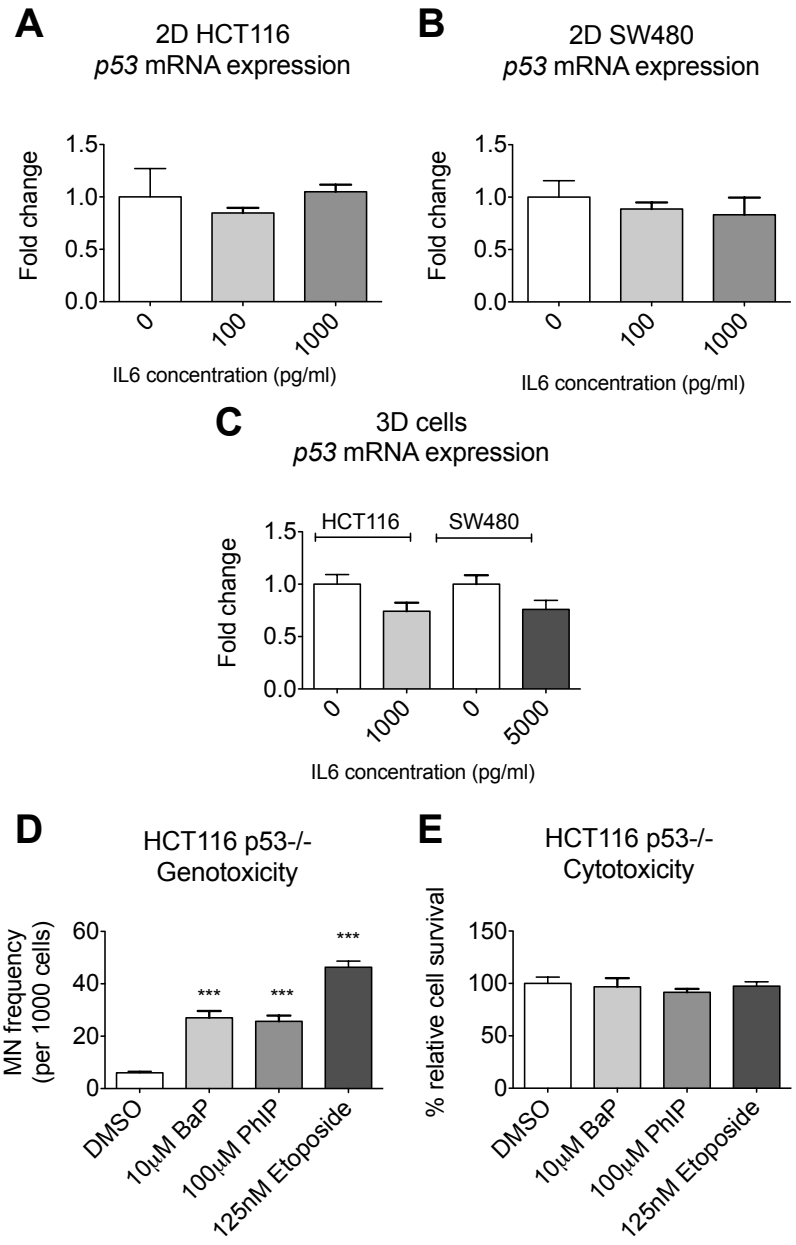

Figure 2.8 IL6-mediated induction of BaP- or PhIP-mediated micronuclei formation is not caused by downregulation of *p53* expression. HCT116 and SW480 cells grown as 2D monolayers (A, B) and 3D spheroids (C) were treated with IL6 and *p53* expression was measured by RT-qPCR. Data were normalised to expression of *GAPDH* and are show relative to control. (D-E) HCT116 p53-/- cells were grown as monolayers and treated for 24 hours with BaP or PhIP. Cells were taken 72 hours post-treatment. Mean micronuclei frequency per 1000 cells (D) and mean cytotoxicity as % of cell survival compared to control (E) were determined following treatment. Significance was calculated using one-way ANOVA with Dunnett post-test comparing treated group to vehicle control (GraphPad Prism 5, ***p<0.001). Error bars represent the SEM for independent cultures (n=3). Adapted from Patel & Gooderham 2015.

2.3.2 Can IL6 regulate *CYP450* expression?

In the current study, I examined the effect of a dose range of IL6 treatment at various time points on *CYP450* expression in CRC cells. A dose-dependent upregulation of *CYP1B1* and *CYP2E1* in both HCT116 and SW480 cells following IL6 treatment was observed (Figure 2.9), and was further confirmed in cells cultured as 3D spheroids (Figure 2.10). However, treatment with IL6 did not significantly alter *CYP1A1* expression (Figure 2.9 A and 2.10 A).

Furthermore, I investigated the effect of pre-treatment with IL6 followed by BaP or PhIP treatment on *CYP1A1* and *CYP1B1* expression, the two CYP isoforms that activate these chemicals, but found no significant changes with IL6 pre-treatment followed by BaP or PhIP compared to BaP or PhIP treatment alone (Figure 2.11). This may be due to the fact that IL6 is removed prior to BaP and PhIP treatment and therefore, *CYP1B1* induction is not sustained following IL6 removal. BaP (but not PhIP) is a known inducer of the AhR pathway that controls *CYP1A1* and *CYP1B1* expression and here, as expected, BaP significantly induced expression of both *CYP1B1* and *CYP1A1*. Similar results were also obtained with cells cultured in 3D (Figure 2.12).

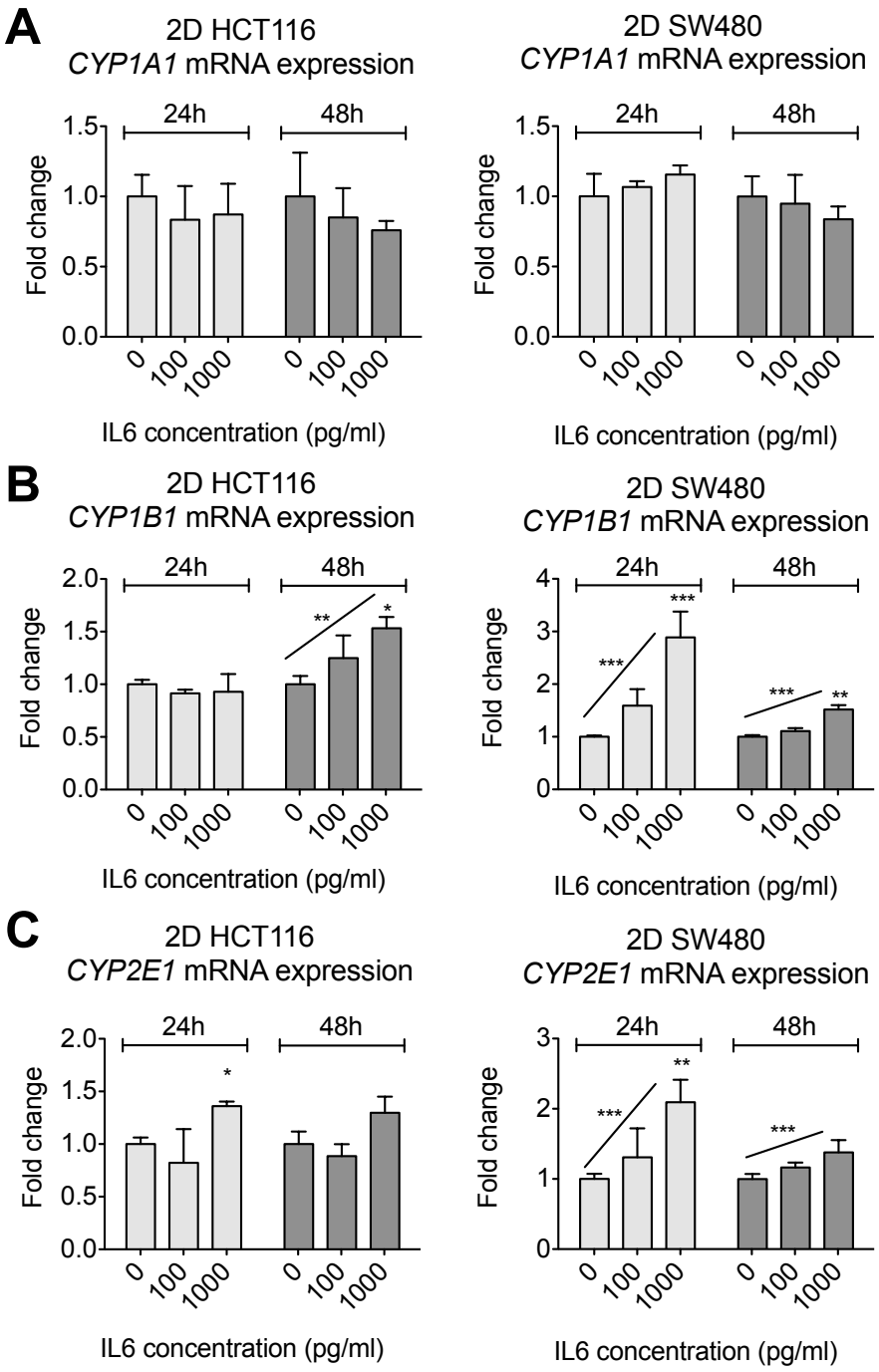

Figure 2.9 IL6 effect on *CYP1A1, 1B1 and 2E1* gene expression. HCT116 and SW480 cells were treated with 0, 100 and 1000pg/ml IL6 for 24 and 48 hours. *CYP1A1* (A), *CYP1B1* (B) and *CYP2E1* (C) expression were measured by RT-qPCR. Data were normalised to expression of *GAPDH* and are shown relative to control. Significance was calculated using one-way ANOVA with a Dunnett post-test comparing treated groups to vehicle control and linear trend analysis (GraphPad Prism 5, *p<0.05, **p<0.01, ***p<0.001). Data are presented as a mean of at least three biological replicates. Error bars represent the SEM for independent cultures. Reproduced from Patel *et al.* 2014.

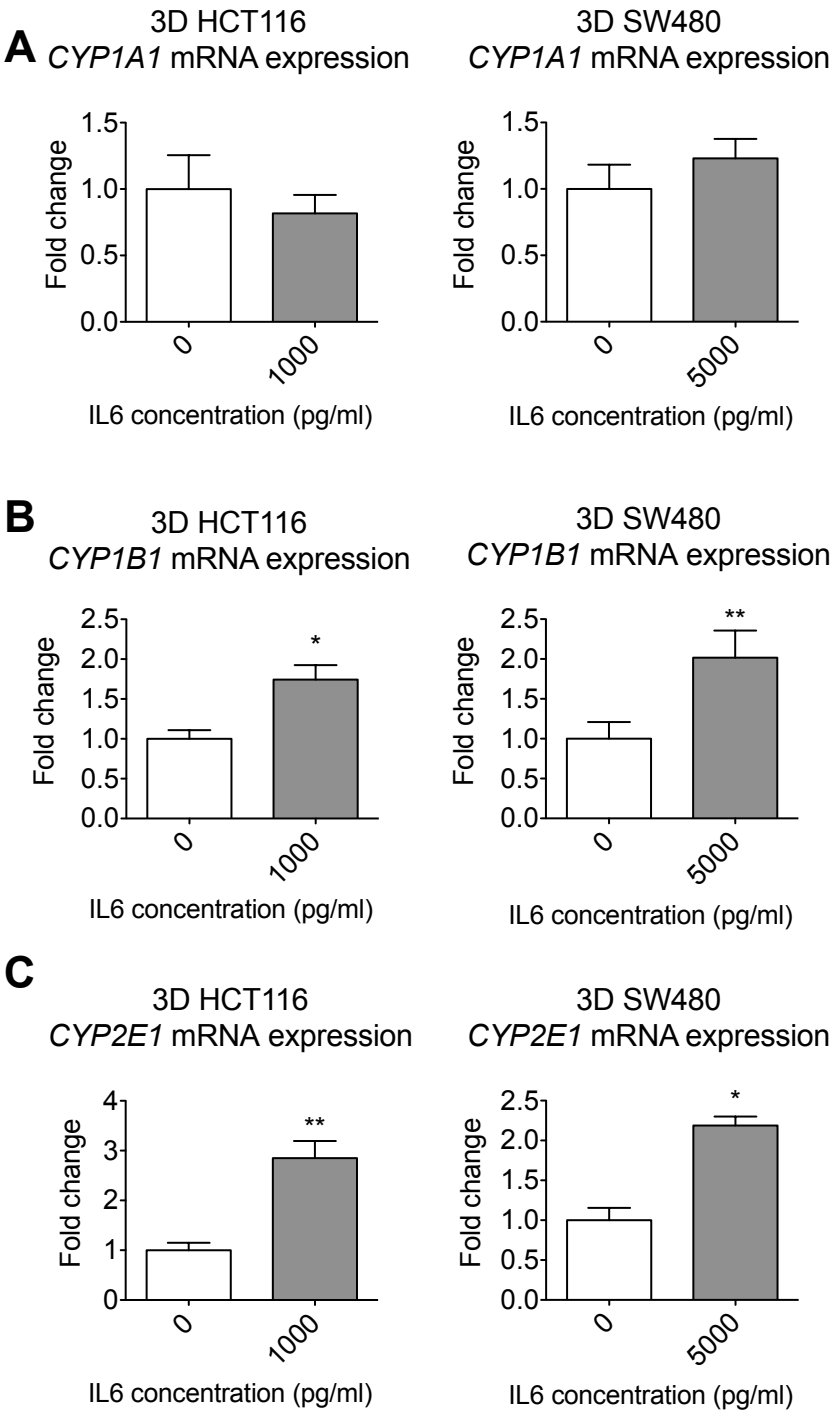

Figure 2.10 IL6 effect on *CYP1A1, 1B1 and 2E1* gene expression in 3D cultures. HCT116 and SW480 cells grown as 3D spheroids were treated with 0, 1000 or 5000pg/ml IL6 for 24 hours. *CYP1A1* (A), *CYP1B1* (B) and *CYP2E1* (C) expression were measured by RT-qPCR. Data were normalised to expression of *GAPDH* and are shown relative to control. Significance was assessed using a Student's *t*-test comparing the treated group to vehicle control (GraphPad Prism 5, $*p<0.05$, $**p<0.01$). Data are presented as a mean of three biological replicates. Error bars represent the SEM. Reproduced from Patel & Gooderham 2015.

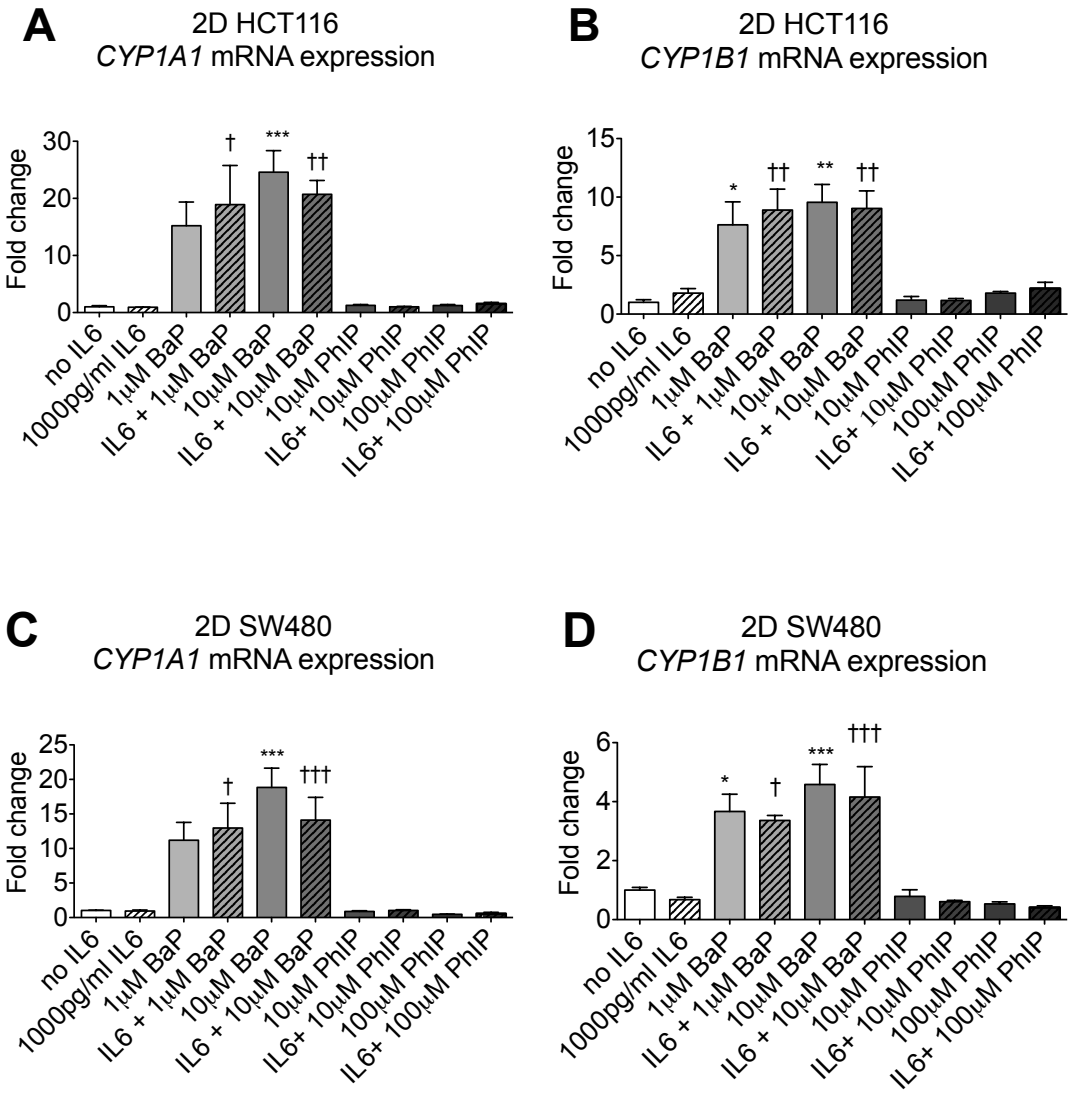

Figure 2.11 *CYP1A1* and *CYP1B1* gene expression following IL6 pre-treatment and BaP or PhIP treatment in 2D cultured HCT116 and SW480 cells. Cells grown as monolayers were pre-treated with 1000pg/ml IL6 for 24 hours (SW480) or 48 hours (HCT116) followed by a 24 hour treatment with BaP or PhIP. *CYP1A1* and *CYP1B1* expression were measured by RT-qPCR. Data were normalised to expression of *GAPDH* and are shown relative to control. Statistically significant differences were calculated using one-way ANOVA with a Dunnett post-test (GraphPad Prism 5) and are shown for comparisons between vehicle *vs* carcinogen treated samples (*p<0.05, **p<0.01, ***p<0.001) and IL6 alone *vs* IL6 pre-treated and carcinogen treated (†p<0.05, ††p<0.01, †††p<0.001). Error bars represent the SEM for independent cultures (n=3). Adapted from Patel & Gooderham 2015.

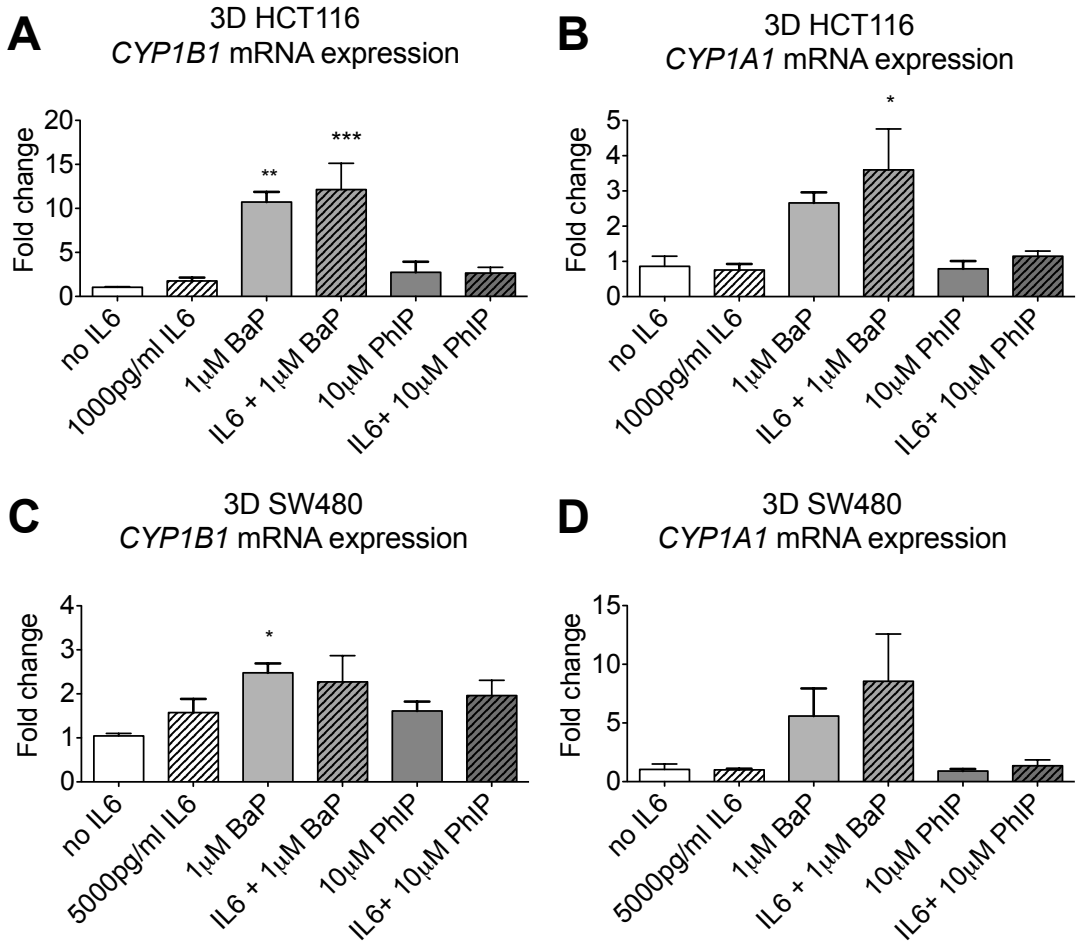

Figure 2.12 *CYP1A1* and *CYP1B1* gene expression following IL6 pre-treatment and BaP or PhIP treatment in 3D cultured HCT116 and SW480 cells. Cells grown as 3D spheroids on Algimatrix were pre-treated with 1000pg/ml IL6 for 24 hours followed by a 24 hour treatment with 1μM BaP or 10μM PhIP. *CYP1A1* and *CYP1B1* expression were measured by RT-qPCR. Data were normalised to expression of *GAPDH* and are shown relative to control. Significance was calculated using one-way ANOVA with a Dunnett post-test comparing treated group to vehicle control (GraphPad Prism 5, *p<0.05, **p< 0.01, ***p< 0.001). Error bars represent the SEM for independent cultures (n=3).

In addition, CYP1A/1B1 activity was measured in 2D HCT116 cells using the EROD assay following IL6, BaP and PhIP treatment (Figure 2.13). No significant changes were observed with IL6 treatment (Figure 2.13 A) likely due to the fact that this assay measures both CYP1A and 1B1 enzyme activities and *CYP1A1* expression is not significantly changed with IL6 treatment. Interestingly, 3D cells appear to have increased EROD activity compared to 2D cells (Figure 2.13 B), which could possibly explain the previously observed increase in MN frequency by BaP and PhIP in 3D cells.

Taken together, these data suggesting that it is the initial induction of *CYP1B1* expression (in the first 24 to 48 hours) by IL6 treatment prior to the addition of the carcinogens that appears to result in increased amounts of activated carcinogens and DNA damage.

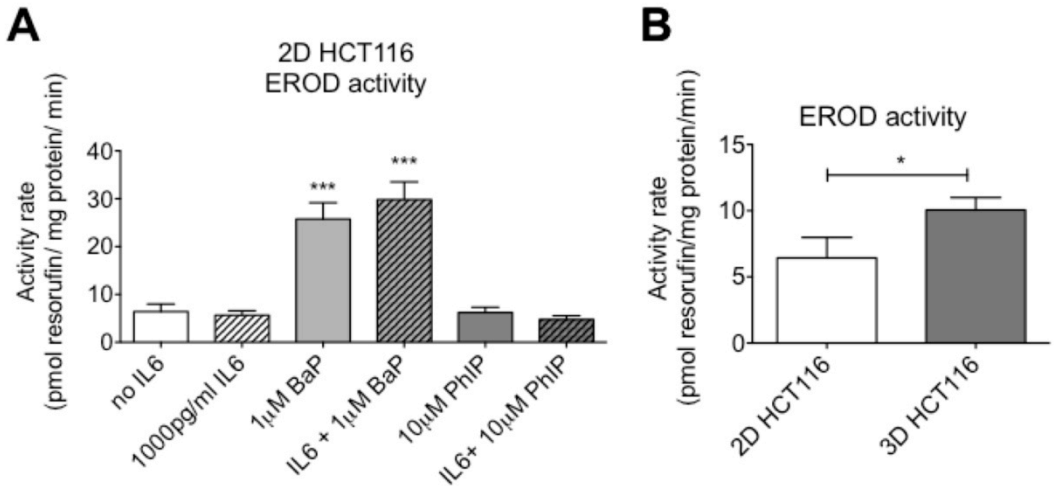

Figure 2.13 CYP1A/1B1 activity in HCT116 cells. (A) Cells grown as 2D monolayers were treated with and without 1000pg/ml IL6 for 48 hours or pre-treated with 1000pg/ml IL6 for 48 hours followed by a 24 hour treatment with 1μM BaP or 10μM PhIP. EROD activity of CYP1A/1B1 enzymes was measured and significance was calculated using one-way ANOVA with a Dunnett post-test comparing treated group to vehicle control (GraphPad Prism 5, ***p< 0.001). (B) EROD activity was measured in 2D and 3D cultured HCT116 cells. Significance was calculated using a Student's *t*-test (GraphPad Prism 5, *p< 0.05). Data are expressed as activity rate in pmol resorufin/mg protein/min. Error bars represent the SEM for at least three independent cultures.

2.3.3 What are the mechanisms involved in IL6-mediated upregulation of *CYP2E1* and *CYP1B1*?

In order to determine the mechanism underlying IL6 induction of *CYP1B1* and *CYP2E1* expression, I examined the different pathways involved in their regulation.

The AhR pathway is the most studied pathway by which *CYP1B1* and *CYP1A1* mRNA expressions are induced whereby activation of the receptor usually following presence of aromatic hydrocarbons such as BaP, results in nuclear translocation, promoter binding and gene transcription. However, *CYP1A1* mRNA expression was not induced upon IL6 treatment, thus the AhR pathway is not likely to be involved in IL6-mediated induction of *CYP1B1*.

MiRNAs that target *CYP1B1* and *CYP2E1* mRNA are another means of regulating expression. MiR27b and miR378 are currently the only validated miRNAs to target *CYP1B1* and *CYP2E1* mRNA expression respectively by direct binding to their 3'UTR (Tsuchiya *et al.* 2006; Mohri *et al.* 2010). I thus investigated the effect of IL6 on miR27b and miR378 and found downregulation of miR27b but no change in miR378 expression in both HCT116 and SW480 cell lines (Figure 2.14 A and C). Furthermore, miR27b expression produced a significant inverse correlation with *CYP1B1* expression (Figure 2.14 D), while miR378 expression did not correlate with *CYP2E1* expression. MiR27b downregulation with IL6 treatment was further confirmed in cells grown as 3D spheroids (Figure 2.15). Transfection of a miR27b inhibitor appeared to result in an upregulation of *CYP1B1* expression, although this result was not statistically significant (Figure 2.16). No significant changes were observed in miR27b expression with IL6 pre-treatment followed by BaP or PhIP compared BaP or

PhIP treatment alone (Figure 2.17), which correlates with what was observed for *CYP1B1* expression (Figure 2.11), suggesting that downregulation of miR27b and modulation of *CYP1B1* mRNA occur in the first 24 to 48 hours of IL6 treatment and are not maintained following IL6 removal. Therefore, these data suggest a possible post-transcriptional mechanism for IL6-mediated regulation of *CYP1B1* *via* miR27b and that regulation of *CYP2E1* does not occur *via* miR378.

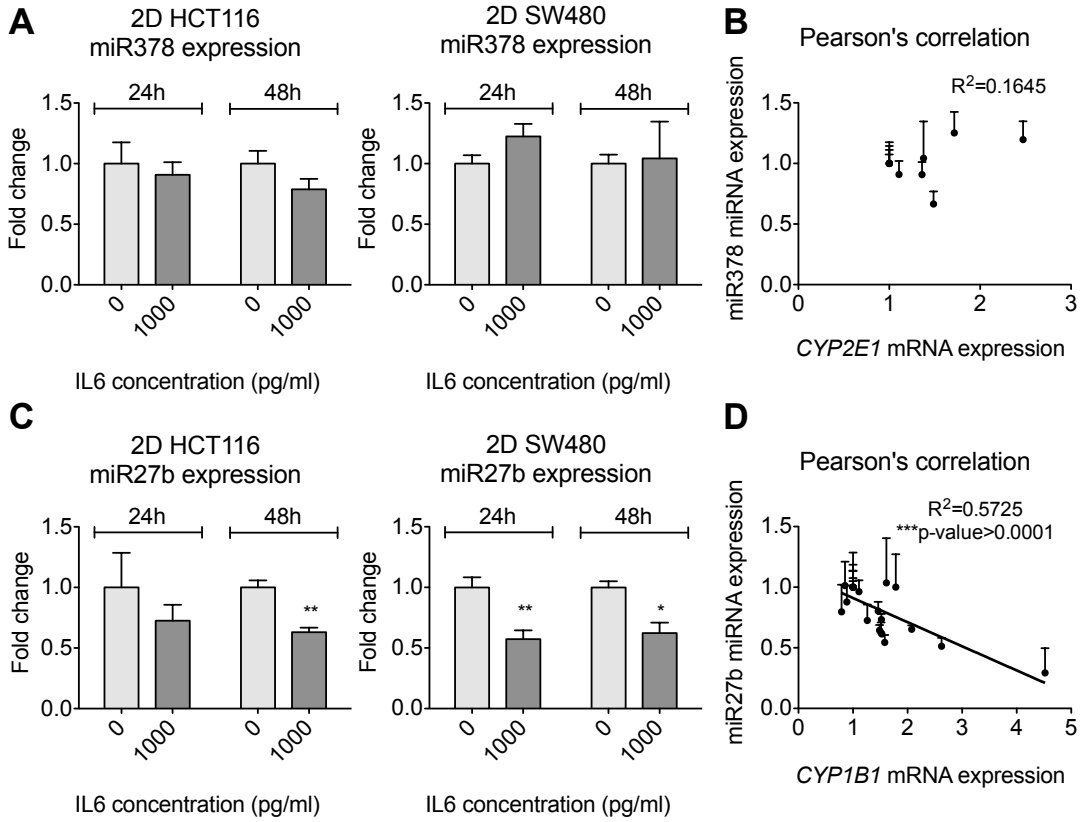

Figure 2.14 MiRNA involvement in IL6-mediated regulation of *CYP1B1* and *CYP2E1* gene expression. HCT116 and SW480 cells grown as monolayers were treated with 1000pg/ml IL6 for 24 and 48 hours. MiR378 (A) and miR27b (C) expression were measured by RT-qPCR. Fold change expression of miR378 was correlated with fold change expression of *CYP2E1* (B) and fold change expression of miR27b with fold change *CYP1B1* expression (D). Data were normalised to expression of U6 RNA. Significance was assessed using Student's *t*-test. Pearson's product-moment correlation coefficient test was used for correlation analysis (GraphPad Prism 5, **p*<0.05, ***p*<0.01). Data are presented as a mean of at least three biological replicates and are shown relative to control. Error bars represent the SEM for independent cultures. Reproduced from Patel *et al.* 2014.

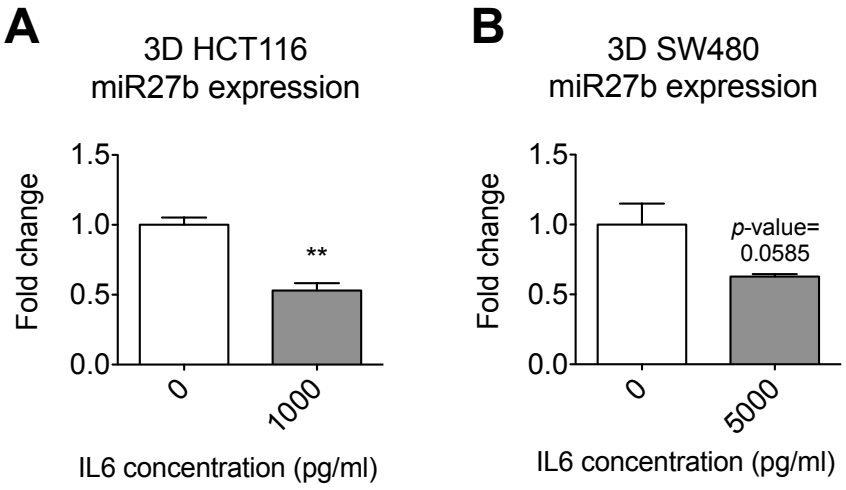

Figure 2.15 MiR27b expression following IL6 treatment in 3D cultures. HCT116 and SW480 cells grown as 3D spheroids were treated with 0 and 1000-5000pg/ml IL6 for 24 hours. MiR27b expression were measured by RT-qPCR in HCT116 (A) and SW480 (B) cells. Data were normalised to expression of U6 RNA and are shown relative to control. Significance was assessed using Student's *t*-test (GraphPad Prism 5, $^{**}p<0.01$). Data are presented as a mean of at least three biological replicates. Error bars represent the SEM for independent cultures.

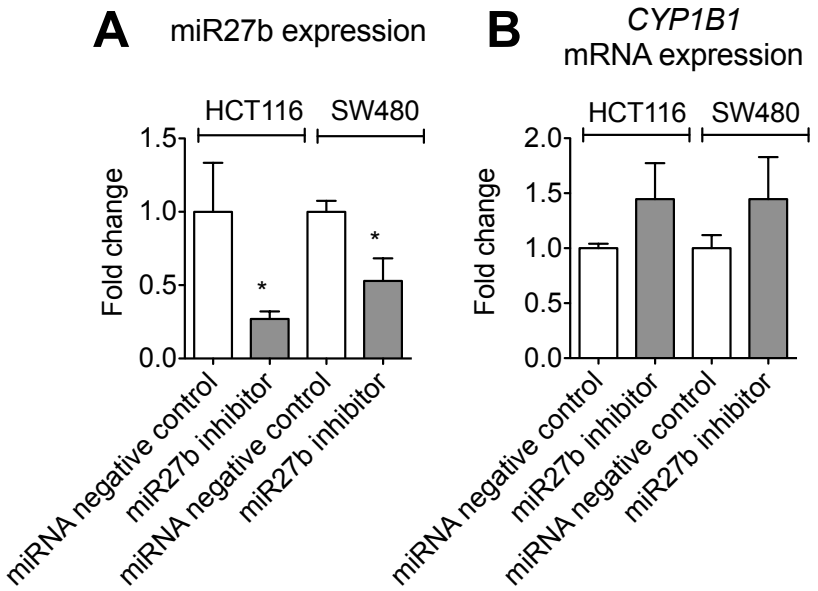

Figure 2.16 MiR27b regulation of *CYP1B1* mRNA in HCT116 and SW480 cells. Cells were transfected with a miR27b inhibitor for 6 hours and expression of miR27b and *CYP1B1* were measure by RT-qPCR. Data were normalised to expression of U6 RNA for miR27b expression and *GAPDH* for CYP1B1 expression, and are shown relative to control. Significance was assessed using Student's *t*-test (GraphPad Prism 5, $^{*}p<0.05$). Data are presented as a mean of at least three biological replicates. Error bars represent the SEM for independent cultures.

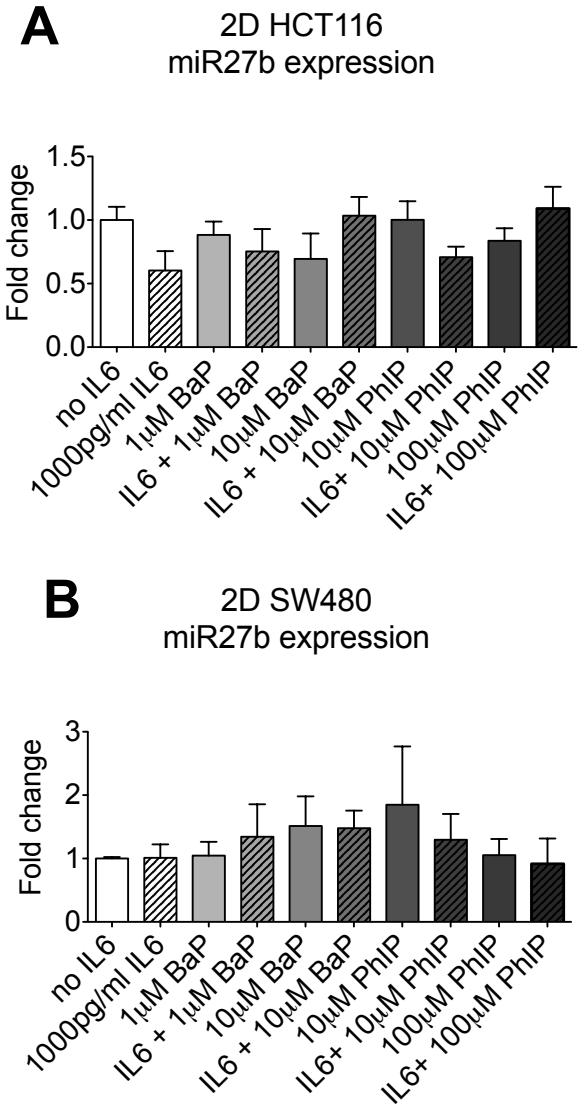

Figure 2.17 MiR27b expression following IL6 pre-treatment and BaP or PhIP treatment. Cells grown as monolayers were pre-treated with IL6 for 48 hours (HCT116- A) or 24 hours (SW480- B) followed by 24 hour treatment with BaP or PhIP. MiR27b expression was measured by RT-qPCR. Data were normalised to expression of U6 RNA and are shown relative to control. Significance was assessed using one-way ANOVA with a Dunnett post-test comparing treated group to vehicle control (GraphPad Prism 5, $*p<0.05$, $**p<0.01$). Error bars represent the SEM for independent cultures (n=3). Adapted from Patel & Gooderham 2015.

IL6 is known to induce the JAK/STAT3 pathway and STAT3 phosphorylation was increased following IL6 treatment (Figure 2.18). I thus investigated whether activation of STAT3 was involved in upregulation of *CYP1B1* and *CYP2E1* by co-

treating cells with IL6 and a STAT3 inhibitor. IL6-mediated induction of *CYP2E1* but not *CYP1B1* was prevented in cells when STAT3 activity was inhibited, suggesting that STAT3 can regulate *CYP2E1* expression (Figure 2.19 A and B). An analysis of the *CYP2E1* promoter region revealed two potential STAT binding sites at positions -36bp and -617bp upstream of the start site (Figure 2.20 A; TFSEARCH ver1.3; Heinemeyer *et al.* 1998). ChIP experiments confirmed that IL6 induced STAT3 binding to both *CYP2E1* regions and binding was prevented when STAT3 was inhibited. Furthermore, binding was higher at the promoter proximal region (position -36bp, CYP2E1 site 2) compared to the distal region (Figure 2.20 B).

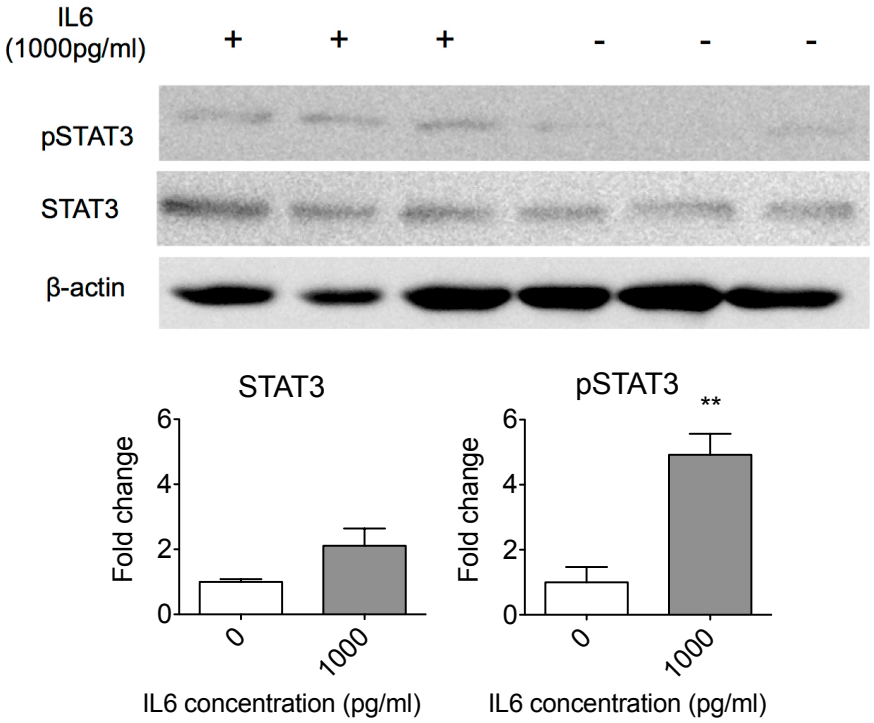

Figure 2.18 Levels of phosphorylated STAT3 are increased upon IL6 treatment. SW480 cells were treated with 1000pg/ml IL6 for 24 hours. STAT3 and phosphorylated STAT3 (pSTAT3) protein expression were determined by immunoblotting and quantified using a Kodak image station 4000MM. Protein expression was normalised to β-actin expression (loading control). Significance was assessed using Student's *t*-test (GraphPad Prism 5, $^{**}p<0.01$). Data are presented as a mean and error bars represent the SEM for independent cultures (n=3).

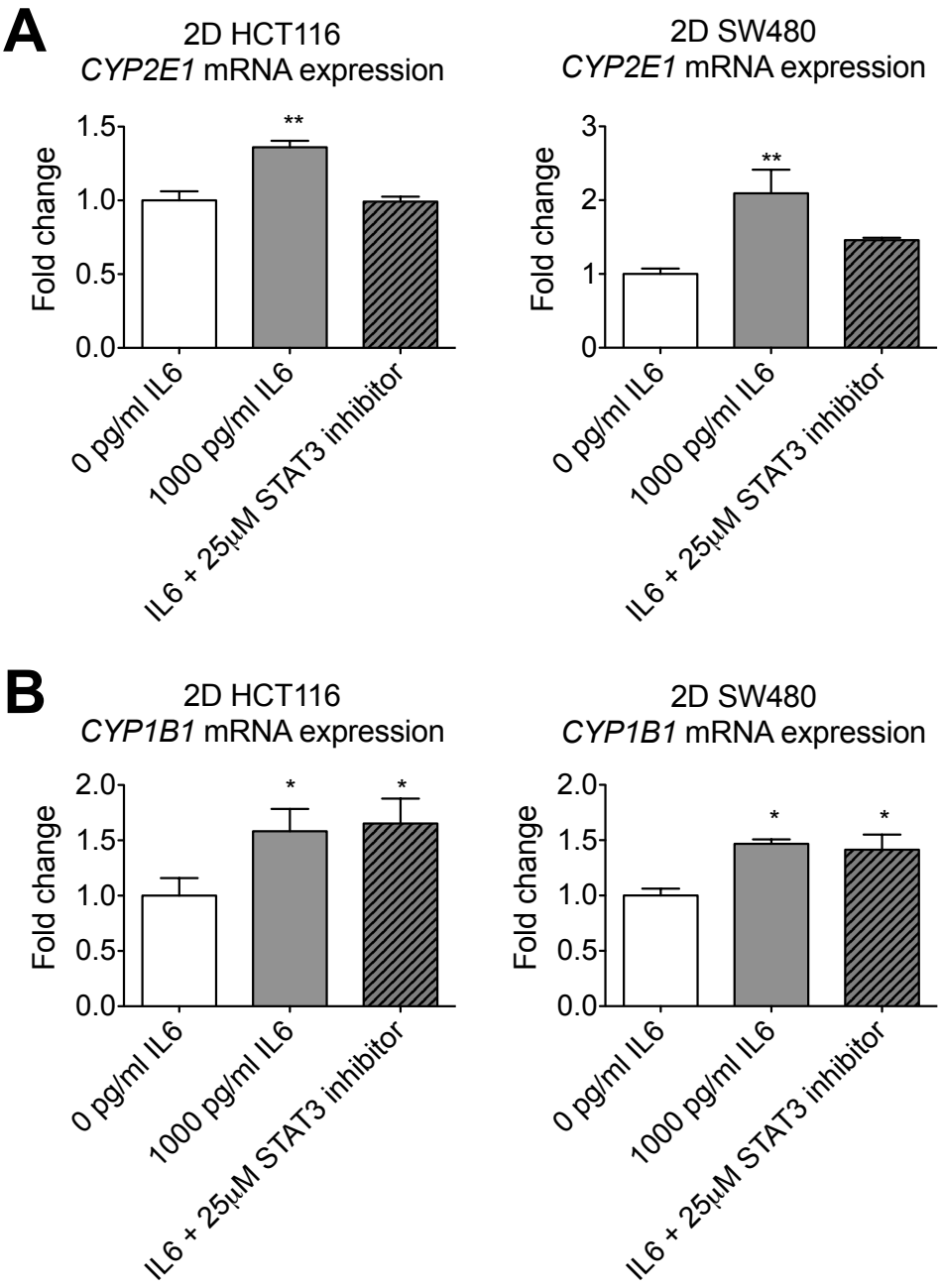

Figure 2.19 STAT3 involvement in IL6-mediated regulation of *CYP1B1* and *CYP2E1* gene expression. HCT116 and SW480 were treated with 1000pg/ml IL6 or IL6 with 25μM STAT3 inhibitor VII for 24 hours (SW480) and 48 hours (HCT116). *CYP2E1* (A) and *CYP1B1* (B) expression was measured by RT-qPCR. Data were normalised to expression of *GAPDH* and are shown relative to control. Data are presented as a mean of at least three biological replicates and error bars represent the SEM for independent cultures. Significance was calculated using one-way ANOVA with a Dunnett post-test comparing treated groups to vehicle control (GraphPad Prism 5, $^{**}p<0.01$). Adapted from Patel *et al.* 2014.

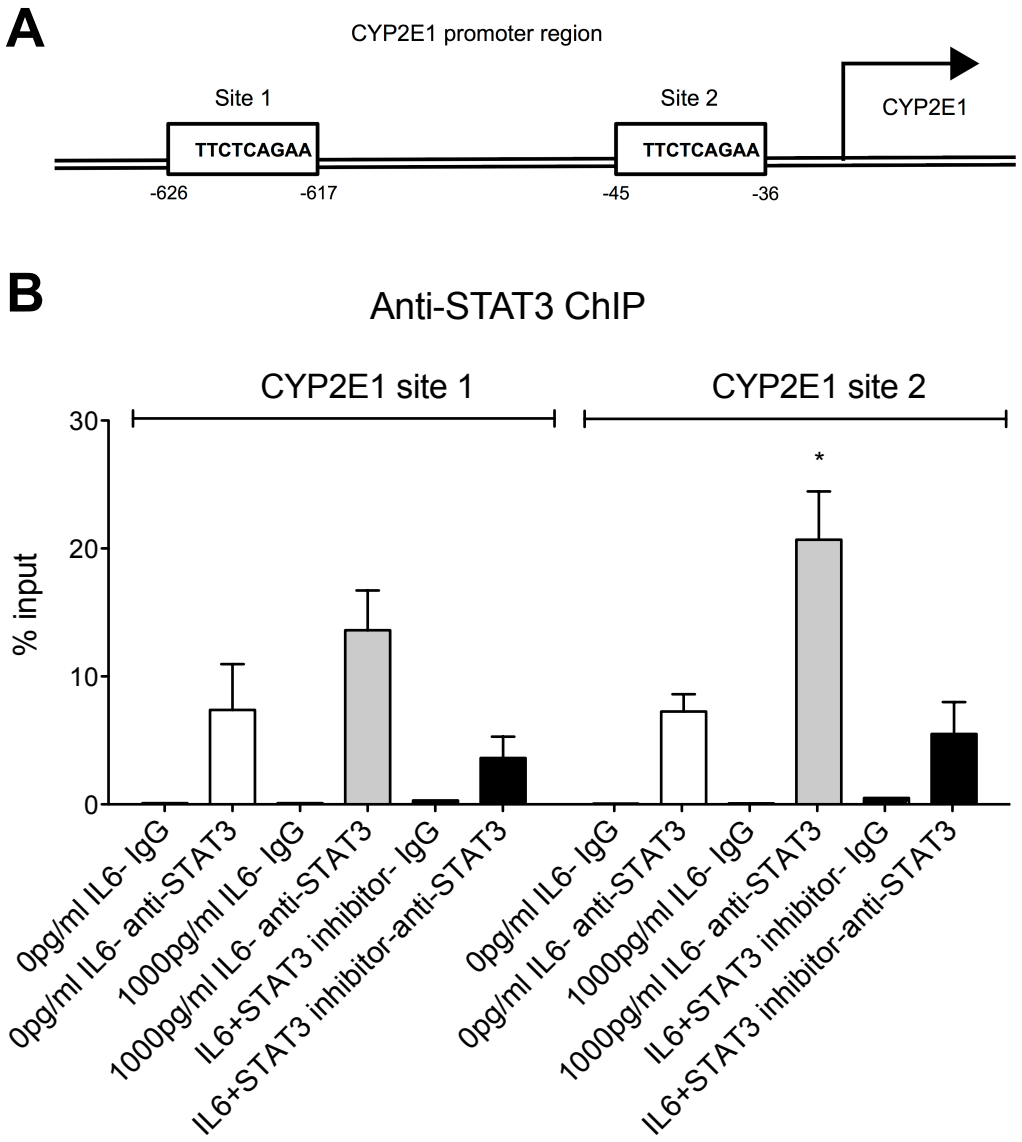

Figure 2.20 STAT3 binds to the *CYP2E1* gene promoter. (A) STAT3 binding sites in the *CYP2E1* promoter region predicted using TFSEARCH ver1.3 (Heinemeyer *et al.* 1998). (B) SW480 cells were treated with 1000pg/ml IL6 or a combination of IL6 and 25μM STAT3 inhibitor VII for 60min and STAT3 binding was measured by ChIP-qPCR using an anti-STAT3 antibody. Data are presented as a mean of at least three biological replicates and error bars represent the SEM for independent cultures. Significance was calculated using two-way ANOVA with a Bonferroni post-test comparing treated groups to vehicle control (GraphPad Prism 5, $*p<0.05$). ChIP experiments were repeated using IgG antibody to control for non-specific binding and performed in duplicate. Adapted from Patel *et al.* 2014.

2.3.4 How does IL6 mediate downregulation of miR27b expression?

Having determined that miR27b downregulation is responsible for IL6-mediated *CYP1B1* upregulation, I next investigated the mechanism involved. MiR27b is located within a miRNA cluster in the *C9orf3* gene (chromosome 9) and its expression has been reported to be regulated through methylation of an adjacent CpG island (Yan *et al.* 2011). As IL6 is also known to promote genome-wide methylation through activation of DNMT1 by AKT-mediated phosphorylation (Hodge *et al.* 2007), the role of DNA methylation in IL6 regulation of miR27b was investigated using 5-aza-2'-deoxycytidine treatment, a potent inhibitor of DNA methylation. When DNA methylation was inhibited, the downregulation of miR27b by IL6 was also prevented in both cell lines blocking the induction of *CYP1B1* expression by IL6 (Figure 2.21). These data therefore suggest that DNA methylation is involved in miR27b downregulation and upregulation of *CYP1B1* by IL6.

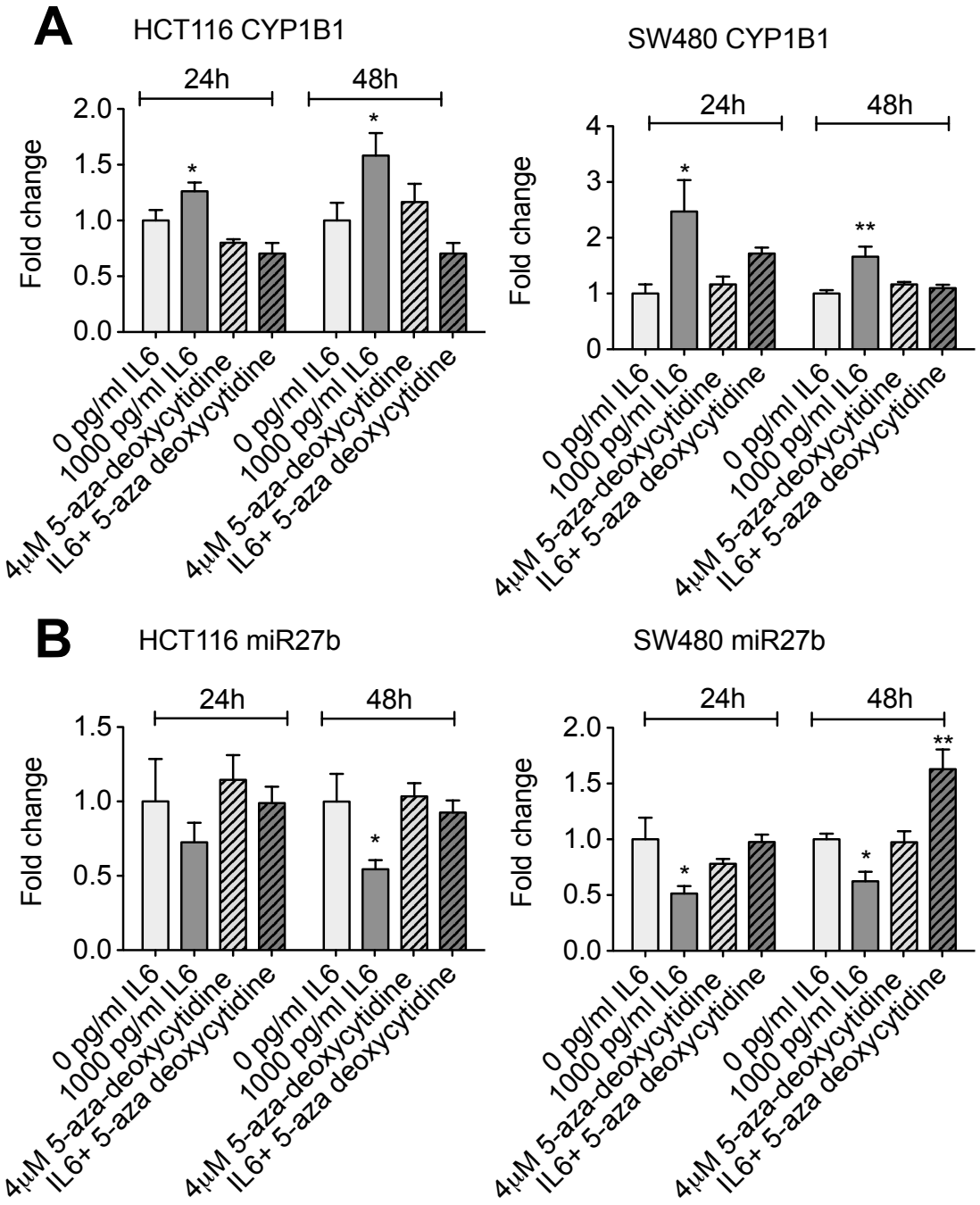

Figure 2.21 DNA methylation involvement in IL6-induced downregulation of miR27b. HCT116 and SW480 cells were treated with 1000pg/ml IL6, 4μM 5-aza-2'-deoxycytidine or IL6 with 5-aza-2'-deoxycytidine for 24 and 48 hours. (A) MiR27b expression was measured by RT-qPCR. (B) *CYP1B1* expression was measured by RT-qPCR. Data were normalised to expression of U6 RNA for miR27b expression and *GAPDH* for *CYP1B1* expression, and are shown relative to control. Significance was calculated using Student's *t*-test and one-way ANOVA with a Dunnett post-test comparing treated groups to vehicle control (GraphPad Prism 5, **p*<0.05, ***p*< 0.01). Data are presented as a mean of at least three biological replicates. Error bars represent the SEM for independent cultures. Adapted from Patel *et al.* 2014.

2.4 Discussion

Inflammation has long been associated with cancer particularly CRC as chronic bowel inflammation is an important risk factor for disease. One of the drivers of this link is thought to be the pro-inflammatory cytokine IL6, which is known to be overexpressed in CRC tumours (Maihöfner *et al.* 2003; Nagasaki *et al.* 2014; Chung *et al.* 2006; Lu *et al.* 2015; Uchiyama *et al.* 2012). Here, I propose a novel role for IL6 as a promoter of dietary carcinogen- induced DNA damage in CRC cells.

In the current study, IL6 was able to promote DNA damage induced by BaP and PhIP, two carcinogens present in meats cooked at high temperatures. To the best of my knowledge, this was the first account of this IL6-mediated effect, however two studies conducted by Dr Lenka Umannová and colleagues have previously reported that TNFα, another pro-inflammatory cytokine, increased BaP-induced DNA adduct formation in rat liver and alveolar type II epithelial cells through induction of CYP1B1 expression (Umannová *et al.* 2011; Umannová *et al.* 2008), suggesting that other pro-inflammatory cytokines may have a similar effect.

I determined DNA damage using the MN assay, which detects double-strand DNA breaks to evaluate genotoxicity. This assay is a regulatory acceptable assay used along with the Ames test (an *in vitro* bacterial gene mutation assay using strains of *Salmonella typhimurium*) as part of a core system used by the UK government to assess genotoxic potential of chemicals *in vitro* (Committee on Mutagenicity of Chemicals in Food Consumer Products and the Environment 2011). However, in the current study, the purpose was to assess genotoxic potential and not to comply with regulatory requirements, and thus results were

not validated using a second method for detecting DNA damage. Indeed, the Ames test would not be appropriate to validate the IL6-mediated effects observed in the MN assay as bacterial organisms lack the endogenous mammalian mechanisms involved in these effects (CYP450 expression, miR27b, STAT3 activation). Other mammalian genotoxicity assays have been shown to have low specificity leading to false positive/negative results (Kirkland *et al.* 2007; Kirkland *et al.* 2005). Recently, the γH2AX assay, a novel test for detecting double-strand breaks in mammalian cells, has emerged as a promising new alternative (Garcia-Canton *et al.* 2012), but it has not been tested on as many compounds as the other standard tests and is not included in the current regulatory guidelines. Nonetheless, had time permitted, it would have been a useful tool to further validate the current MN assay findings.

I suggest that IL6 promotes dietary carcinogen-mediated DNA damage through induction of *CYP1B1* expression, a member of the CYP450 family of enzymes known to activate BaP and PhIP. I also found that IL6 increased *CYP2E1* expression, another CYP450 protein known to activate dietary carcinogens such as acrylamide (Ghanayem *et al.* 2005). CYP450s play a crucial role in activation of xenobiotic chemicals such as pro-carcinogens and drugs, as well as in tumour development and progression (Murray *et al.* 1997; Gooderham *et al.* 2007; Braeuning *et al.* 2011; Kasai *et al.* 2013; Rodriguez & Potter 2013), therefore it is important to understand the mechanisms underlying their regulation. Previous studies on IL6 regulation of CYP450 enzymes present conflicting reports. Generally IL6 is thought to have an inhibitory effect in hepatic cells (Abdel-Razzak *et al.* 1993; Jover *et al.* 2002; Hakkola *et al.* 2003), however a few studies in other cell types have shown increased expression of CYP450 in

response to IL6 (Smerdová *et al.* 2014; Tindberg *et al.* 1996; Kurzawski *et al.* 2012), which are in agreement with the data presented here in CRC cells. Therefore, this suggests that IL6 effect on CYP450 may be tissue-dependent and prior to the current study, there were no previous reports of this effect in CRC cells. Furthermore, the majority of these studies used much higher doses of IL6 (up to 50ng/ml), while here lower doses of IL6 (0-5000pg/ml) were used that are within the range secreted by stromal cells in the TME (up to 8000pg/ml; Nagasaki *et al.*, 2014).

Kurzawski *et al.* have shown that *CYP1B1* expression but not *CYP1A1* is increased in response to IL6 and they established that this regulation is independent of the AhR pathway but they did not propose an alternate mechanism (Kurzawski *et al.* 2012). In the current study, I demonstrate that miR27b, a miRNA known to target CYP1B1, is downregulated in response to IL6 in a mechanism involving DNA methylation, resulting in *CYP1B1* upregulation by IL6. A CpG island located near the miR27b gene has been shown to be methylated and regulate miR27b expression (Yan *et al.* 2011), and my findings of IL6-mediated regulation of miR27b could be further validated by investigating DNA methylation at that site following IL6 treatment using bisulphite-sequencing techniques or qPCR methylation kits. Furthermore, IL6 has been shown to promote global DNA methylation changes through activation of DNMT1 by AKT (Hodge *et al.* 2007), knockout studies could be used in order to confirm whether DNMT1 and PI3K/AKT pathway activation are involved in the observed effects. While miR27b and IL6 have both been shown to be involved in inflammation-related pathways (Jin *et al.* 2013; Lee *et al.* 2012), direct regulation of miR27b by IL6 has not been previously reported.

Additionally, IL6 overexpression has been reported *in vivo* in CRC tissue (Maihöfner *et al.* 2003; Chung *et al.* 2006; Lu *et al.* 2015; Uchiyama *et al.* 2012) whereas miR27b has been shown to be downregulated (Table 1, section 1.3; Yang *et al.* 2009), suggesting that IL6 may be involved in downregulating miR27b expression *in vivo* as well.

Furthermore, CYP2E1 expression regulation is not yet well understood and here I propose a transcriptional mechanism *via* direct STAT3 binding to the *CYP2E1* promoter region in response to IL6 treatment. This was demonstrated using a ChIP assay, which showed that following IL6 treatment, STAT3 binds to site 2 of the CYP2E1 promoter with more affinity than site 1 (Figure 2.20). The role of these binding sites could be further investigated by deleting each of the sites and determining *CYP2E1* expression with IL6 treatment or by performing reporter gene assays with the CYP2E1 promoter STAT3 binding sites. CYP2E1 plays a crucial role in drug bioavailability (Koop 1992) and thus changes in CYP2E1 expression may lead to modification in drug response, therefore it is crucial to understand how this enzyme is regulated.

A STAT3 inhibitor was used to investigate STAT3 involvement in the IL6 mediated effects observed in these studies. When the inhibitor was added along with IL6, STAT3 binding was prevented suggesting that the inhibitor was effective at preventing STAT3 activation by IL6. An inhibitor only control was not included in these studies as phosphorylated STAT3 levels are very low in the absence of IL6 treatment (Figure 2.18). However, the lack of an inhibitor only control means any non-specific effects of the inhibitor are not accounted for in these experiments.

When comparing different cell culture methods, we observed that IL6-mediated

effects on promoting BaP- and PhIP-induced DNA damage were not as pronounced in 3D cultures as compared to 2D cultures. It has generally been shown that a decrease in drug sensitivity is observed in cells cultured in 3D compared to 2D (Li *et al.* 2008; Doillon *et al.* 2004) likely due to the differences in level of exposure caused by the architecture of the spheroid, thus 3D culture is thought to better recapitulate *in vivo* responses. In addition, 3D cells without IL6 pre-treatment had a higher level of DNA damage in response to BaP and PhIP but not etoposide compared to 2D cells, likely due to the increased CYP1A/1B1 activity observed in 3D cells; this may also explain the reduced ability for IL6 to further promote BaP/PhIP activation and DNA damage in 3D cells.

In critically evaluating the limitations of the current study, future studies using a STAT3 inhibitor only control and validation of current findings using other experimental techniques and *in vivo* studies are indicated. Despite this, the IL6-mediated effects were observed in two different *in vitro* culture systems (2D and 3D) as well as in two different CRC cell lines (HCT116 and SW480), thus providing some validation to the current findings. While these findings need to be further developed to demonstrate their significance *in vivo*, they do provide a potential mechanistic insight into the link between diet, inflammation and CRC. If these data do indeed prove to be relevant *in* vivo, they would also suggest that drugs activated by CYP1B1 or CYP2E1 could be a potential novel therapeutic strategy for CRC patients that have high levels of IL6 at the tumour site and that dietary prevention methods for patients with an inflammatory bowel condition could have the potential to reduce their CRC risk.

2.5 Summary

Here, I investigated the effect of IL6 on DNA damage caused by dietary pro-carcinogens in CRC cells. Pre-treatment with IL6 enhanced BaP- and PhIP-induced MN formation while IL6 on its own was not genotoxic. IL6 affected the activation pathway of the pro-carcinogens by inducing *CYP1B1* expression potentially through repression of miR27b in a mechanism involving DNA methylation (Figure 2.22). *CYP2E1* expression was also induced by IL6 possibly through a STAT3-meditated mechanism (Figure 2.22). Increased levels of CYP1B1 and CYP2E1 in the cell along with presence of dietary carcinogens could result in increased quantities of genotoxic metabolites, thus resulting in DNA damage and CRC progression. While these data need to be further validated using further *in vitro* and *in vivo* experimentation, the discovery of this potential novel pathway provides further understanding of the possible mechanisms by which IL6 can promote carcinogenesis. Previous reports of IL6-mediated effects (reviewed in section 1.4) suggest that other pathways are also likely to be involved. The next chapter (Chapter 3) focuses on these other pathways by investigating the role IL6 plays in altering behaviour of CRC cells.

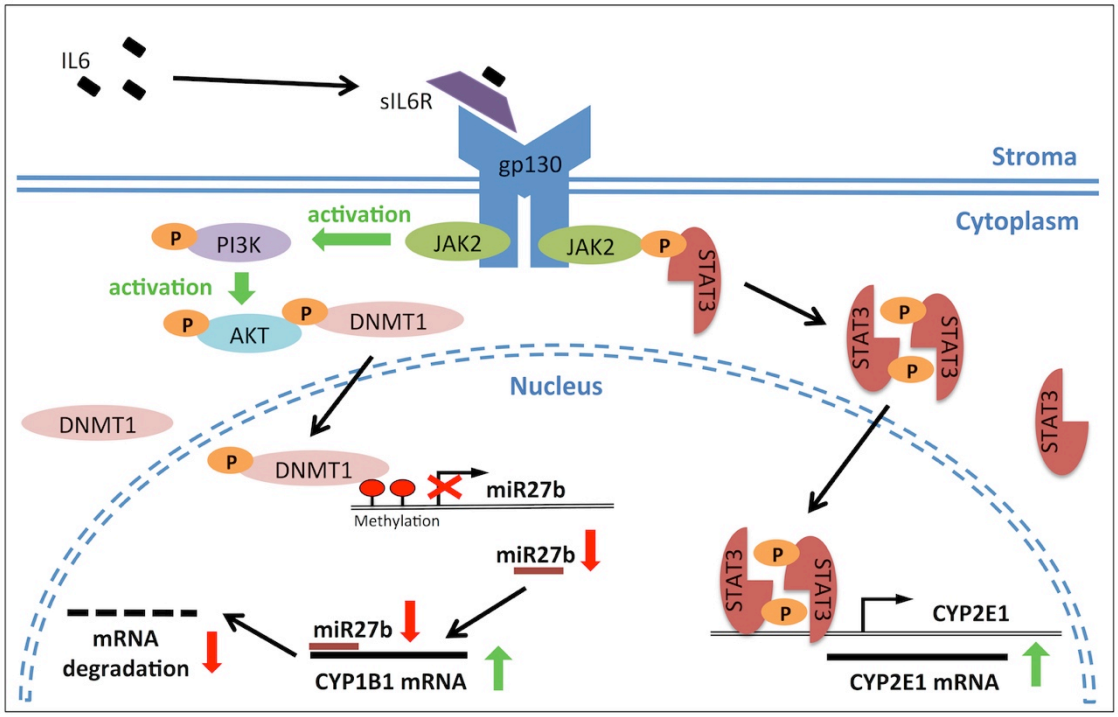

Figure 2.22 Proposed mechanism of CYP1B1 and CYP2E1 regulation by IL6 in CRC cells. IL6 present in the TME binds to the soluble IL6 receptor (sIL6R), which interacts with transmembrane protein gp130 leading to activation of JAK/ STAT3 and PI3K/AKT pathways. Activated STAT3 forms a homodimer, translocates to the nucleus and binds to the *CYP2E1* promoter region, thus inducing its transcription. Activated AKT phosphorylates DNMT1 leading to its nuclear translocation and DNA methylation at a CpG island located near miR27b. Lower expression of miR27b results in increased *CYP1B1* mRNA expression. Reproduced from Patel *et al.* 2014.

- Chapter 3 -

Interleukin-6 promotes behavioural changes in colorectal cancer cells.

N.B. Results from this chapter have been included in the following peer-reviewed publication:

Patel, S.A.A. and Gooderham, N.J., 2015. Interleukin-6 mediates crosstalk between immune and cancer cells via miR21 and miR29b. Molecular Cancer Research (In Press).

3.1 Introduction

Metastatic CRC is responsible for the high disease mortality rate and due to the aggressiveness of the disease, metastases often occur before local growth produces symptoms (Wang *et al.* 2012); thus early detection remains challenging. Further understanding the mechanisms that promote CRC metastasis is key to improving disease outcome.

The formation of metastases involves the primary tumour invading adjacent tissues, entering the systemic circulation and successfully establishing micrometastases at distant sites (often in the liver). This multi-step process requires overexpression of a number of metastasis-promoting molecules such as MMP2 (Kesanakurti *et al.* 2013; Mott & Werb 2004; Azzam *et al.* 1993), Cathepsin D (CTSD; Ahmad *et al.* 2012; Rochefort *et al.* 1990) and VEGF (Brown *et al.* 1997) as a result of the tumour cells undergoing further molecular changes.

Presence of pro-inflammatory cytokine IL6 in the TME is thought to promote cancer progression (Nagasaki *et al.* 2014; Waldner *et al.* 2012). IL6 induces proliferation and invasion of a variety of cancer cell types (Schneider *et al.* 2000; Hsu & Chung 2006; Becker *et al.* 2005). IL6 is known to activate the tumour-promoting STAT3 transcription factor *via* JAK resulting in expression of a number of tumour-promoting genes and miRNAs (Johnson *et al.* 2012; Wei *et al.* 2003; Waldner *et al.* 2010).

MiRNAs are dysregulated in CRC and are thought to play a crucial role in promoting tumour metastasis. MiR135b is upregulated in malignant CRC cells and has been shown to target *APC* gene expression, an important tumour-

suppressor gene silenced during CRC initiation (Nakamura *et al.* 1992; Fearon & Vogelstein 1990). MiR21 is commonly overexpressed in numerous diseases including IBD and CRC (Asangani *et al.* 2008), and targets pro-apoptotic and tumour-suppressor genes (Asangani *et al.* 2008; Liu *et al.* 2012; Li *et al.* 2014); its expression is reported to be induced by STAT3 transcription factor signalling (Yang *et al.* 2010). Let7a is a tumour-suppressor miRNA that is downregulated in multiple cancer types including CRC (Akao *et al.* 2006), and has been shown to target IL6 mRNA (Iliopoulos *et al.* 2009). MiRNAs are therefore intimately involved in the regulation of the cancer cell phenotype as well as IL6 signalling, thus miRNAs may underlie the strong link between IL6 and CRC progression.

In the current chapter, I have investigated the effect of IL6 on CRC cell behaviour and the potential underlying molecular mechanisms using *in vitro* 2D and 3D cell culture methods. My data suggest that IL6 promotes metastatic behaviour in the CRC cells, particularly in the HCT116 cell line. Changes in gene and miRNA expression appear to be involved in this process.

3.2 Materials and Methods

3.2.1. Cell culture

Human HCT116 and SW480 cells were cultured in 2D and 3D culture as previously described (section 2.2.1).

3.2.2. Cell proliferation assay

Viable cells were quantified using AlamarBlue (Invitrogen, Life technologies) according to the manufacturer's protocol. Briefly, cells were seeded in a 24-well plate at a density of $1x10^4$ cells per well. AlamarBlue (10% of final volume) was added at 24 hour intervals. Under these conditions, AlamarBlue is enzymatically converted to the fluorescent product resorufin, which can be measured in a spectrofluorimeter. The enzymatic conversion to resorufin is proportional to cell number. Fluorescence (excitation 560nm/ emission 590 nm) was read in a Fluostar plate reader (BMG Labtech) after 1 hour incubation at 37°C. Background fluorescence was determined as 10% AlamarBlue in culture medium. Results are expressed as fold change compared to the vehicle control.

3.2.3. Wound-healing assay

HCT116 and SW480 CRC cells are able to proliferate in dextran-coated charcoal-stripped FBS supplemented culture medium. One hundred thousand cells/well were plated in 24-well plates and grown for 72 hours in culture medium supplemented with 5% dextran-coated charcoal-stripped FBS until confluent. Cells were wounded using a sterile tip, washed 3 times with PBS, and 1ml of culture medium supplemented with 1% dextran-coated charcoal-stripped FBS containing IL6 treatment was added to each well. Pictures were taken at 0, 24 and 72 hours (10x magnification). A grid placed underneath the plate was

used to normalise width of the channels and 3 pictures were taken per channel for wound width measurements. Wound width for each well was measured and normalised to the width of 1 square on the grid. The percentage migration was calculated as follows:

t= time elapsed since wound created

x(h)= percentage of wound size at t=h hours

= (average width of channel t=h hours/ grid width) / (average width of channel t=0 hours/ grid width) *100

(at t= 0 hour, x(0)= 100%)

% migration(h)= [1-x(h)]*100

% migration(h)= [1-((average width of channel t=h hours/ grid width) / (average width of channel t=0 hours/ grid width))]*100

Results are expressed as fold change compared to the vehicle control.

3.2.4. Transwell migration and invasion assays

For the migration assay, $3x10^4$ cells per well were plated in 100μl of culture medium supplemented with 1% dextran-coated charcoal-stripped FBS in the upper chamber of a 96-transwell insert system with 8μm pores (Figure 3.1, BD Falcon, Oxford, UK). In the lower chamber, 100μl of culture medium containing 10% FBS was added as a chemoattractant. For the invasion assay, 20ul matrigel was added to upper chamber and left to set at room temperature prior to addition of the cells. In both cases, treatment was added to the upper chamber and cells were left to migrate to the lower chamber for 72 hours. The cells were then removed from the upper chamber using a moist cotton swab and cells in the lower chamber were incubated with 10% AlamarBlue (Invitrogen, Life technologies) for 2 hours at 37°C to quantify them. Results are expressed as fold change compared to vehicle control.

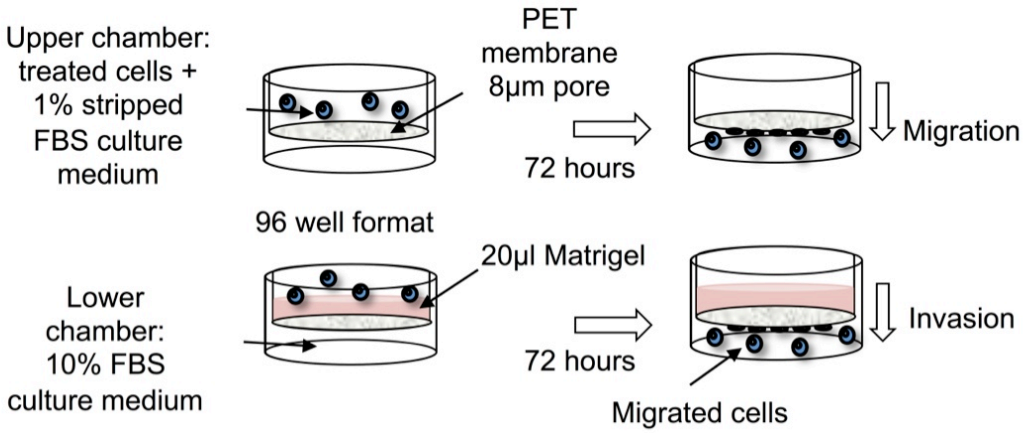

Figure 3.1 Transwell migration and invasion assay method.

3.2.5. IL6 treatment and STAT3 inhibition

Treatments were performed as previously described in section 2.2.2.

3.2.6. RNA extraction

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Life technologies) as previously described (section 2.2.4).

3.2.7. Reverse transcription and qPCR

Reverse transcription and qPCR were performed as previously described (section 2.2.5). Details of primers used are included in Appendix A.

3.2.8. Transfection of miRNA mimics

MiRNA mimics were obtained from miRIDIAN mimics (Thermo Fisher Scientific, Cramlington, UK) and transfected into the cells using Lipofectamine 2000 (Invitrogen, Life technologies) according to the manufacturer's protocol. Briefly, cells were seeded at a density of $1x10^5$ cells per well of a 24-well plate and culture medium was replaced with 400µl/well of Opti-MEM (GIBCO, Life technologies) prior to the addition 150µl/well of Opti-MEM containing 8µl of Lipofectamine 2000 reagent and 2.5µl of 20µM stock of miRNA mimic or

miRIDIAN miRNA negative control. Cells were incubated with the transfection complexes for 24 hours prior to harvest.

3.2.9. Tissue specimens

Tissue samples were kindly provided by Dr Hutan Ashrafian from the St Mary's Biobank (Imperial College London, UK) and ethical approval was obtained from the Imperial College Research Ethics Committee. The study was conducted according to the Helsinki guidelines. Surgical specimens of primary tumours and adjacent normal colon were taken at operation with informed consent from 7 patients with histologically verified colorectal cancer. For all patients, tissue sections of both malignant and adjacent normal (taken outside of the tumour margin) were provided. Tissue samples were homogenised, protein was extracted using RIPA buffer (Sigma-Aldrich) according to the manufacturer's protocol and RNA was extracted using TRIzol reagent (Invitrogen, Life technologies) as described previously (section 2.2.4). IL6 was quantified from protein samples using the MSD ultra-sensitive multiplex human pro-inflammatory cytokine kit (kindly gifted by Dr Hector Keun, Imperial College London, UK) according to the manufacturer's protocol using the MSD Sector Imager 2400 (MSD, Rockville, Maryland, USA), while gene and miRNA expression was determined from the RNA samples using reverse transcription and qPCR as previously described (section 2.2.5).

Statistical analysis

In vitro cell culture. Statistical significance was assessed as previously described (section 2.2.10). *Tissue samples.* Significant differences between tumour and normal tissue were calculated using a Mann-Whitney test (GraphPad Prism 5).

3.3 Results

3.3.1 Can IL6 alter CRC cell behaviour?

The ability for cancer cells to proliferate, migrate and invade surrounding tissues is crucial for cancer progression and metastasis. IL6 is reported to influence metastatic behaviour, therefore the effect of a dose range of human recombinant IL6 (0-20,000pg/ml) was determined on behaviour of CRC cell lines HCT116 and SW480 *in vitro*.

Chronic treatment with IL6 promoted cell proliferation; however, HCT116 cells were more susceptible to IL6 treatment compared to SW480 cells. In the latter, the effect was only observed following 5 days of chronic treatment while a significant increase in proliferation after 48 hours was observed in HCT116 cells (Figure 3.2). Furthermore, these effects were only apparent at the highest dose of IL6 (20,000pg/ml). Addition of a STAT3 inhibitor prevented this IL6-mediated effect in both cell lines, suggesting that STAT3 signalling is likely to be involved in the observed proliferative response (Figure 3.2).

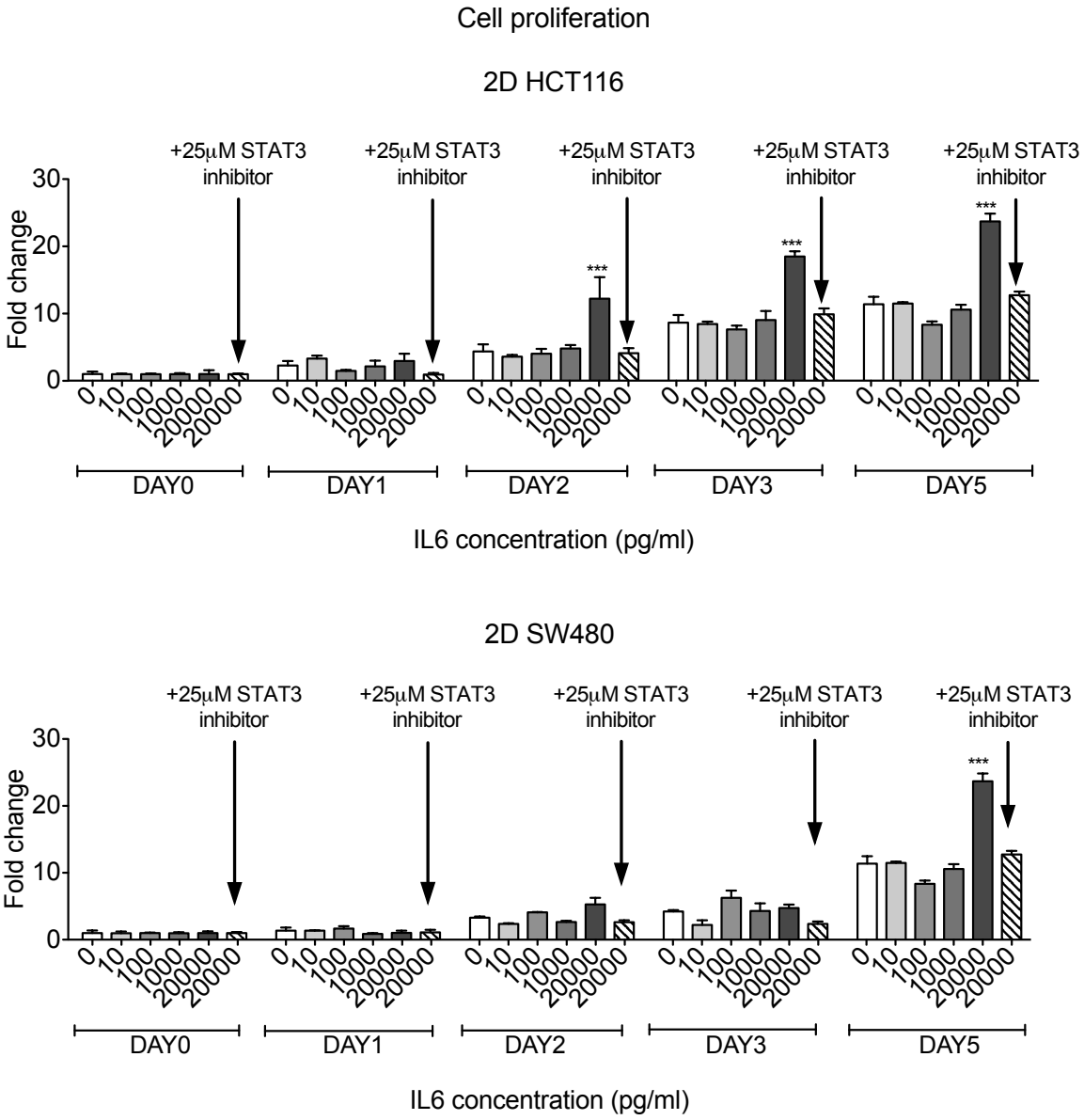

Figure 3.2 Cell proliferation in response to IL6 treatment. HCT116 and SW480 cells were treated daily for 5 days with a dose range of IL6 (0-20,000pg/ml). Cell proliferation was determined using AlamarBlue. Data are expressed as fold change compared to vehicle control (0pg/ml IL6). Significance was calculated using two-way ANOVA followed by a Bonferroni post-test (GraphPad Prism 5, ***p<0.001, **p<0.01, * p<0.05). Error bars represent the SEM for independent cultures (n=3).

In addition, cell migration and invasion in response to IL6 treatment was assessed in both cell lines grown in 2D and 3D cell culture. Cell migration was investigated using a wound-healing assay. Treatment with IL6 significantly increased cell motility in both HCT116 and SW480. However, the latter responded only to doses >200pg/ml after 72 hours (Figure 3.3 C and D) while HCT116 cell migration was significantly increased with ≥40pg/ml after a 24 hour treatment and with ≥10pg/ml after 72 hours (Figure 3.3 A and B). The effect IL6 treatment in HCT116 cells was further confirmed using transwell migration and invasion assays where the cells had increased motility with ≥5pg/ml IL6 (Figure 3.4 A), whereas IL6 failed to produce a significant response in SW480 cells (Figure 3.4 B). Inhibiting STAT3 signalling prevented the IL6-mediated increase in migration and invasion in HCT116 cells (Figure 3.4 A). Moreover, a bell-shaped dose response curve for HCT116 migration and invasion was observed with IL6 treatment. This response was not caused by a drop in cell viability at the higher treatment doses since cell proliferation at those doses was unaffected by IL6 treatment (Figure 3.2). IL6 treatment has been shown to produce a bell-shaped dose response (van Dam *et al.* 1993; Wang *et al.* 2004), as signal transduction does not rely solely on ligand concentration but requires multivalent ligand binding to induce receptor crosslinking (in the case of IL6, signalling is mediated by dimerization of gp130) for activation of downstream signalling.

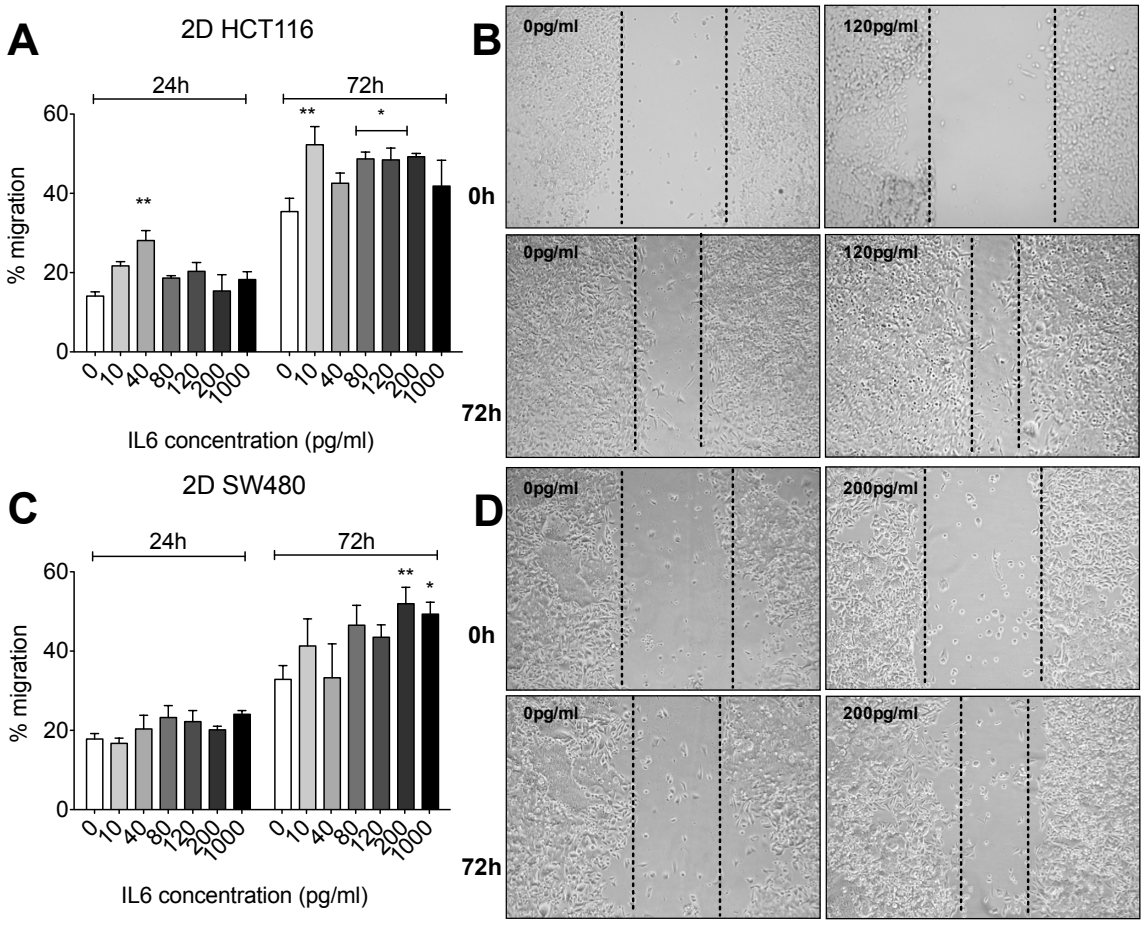

Figure 3.3 Effect of IL6 on wound healing in HCT116 and SW480 cells. (A and C) Wound assays were performed over 72 hours using HCT116 and SW480 cells treated with IL6 (0-1000pg/ml). (B and D) Pictures of wounded HCT116 (B) and SW480 (D) cells taken at 0 or 72 hours with or without IL6 treatment (10x magnification). Data are expressed as fold change compared to vehicle control (0pg/ml IL6). Significance was calculated using one-way ANOVA with a Dunnett post-test comparing treated groups to vehicle control (GraphPad Prism 5, *p<0.05, **p<0.01, ***p<0.001). Error bars represent the SEM for independent cultures (n=3).

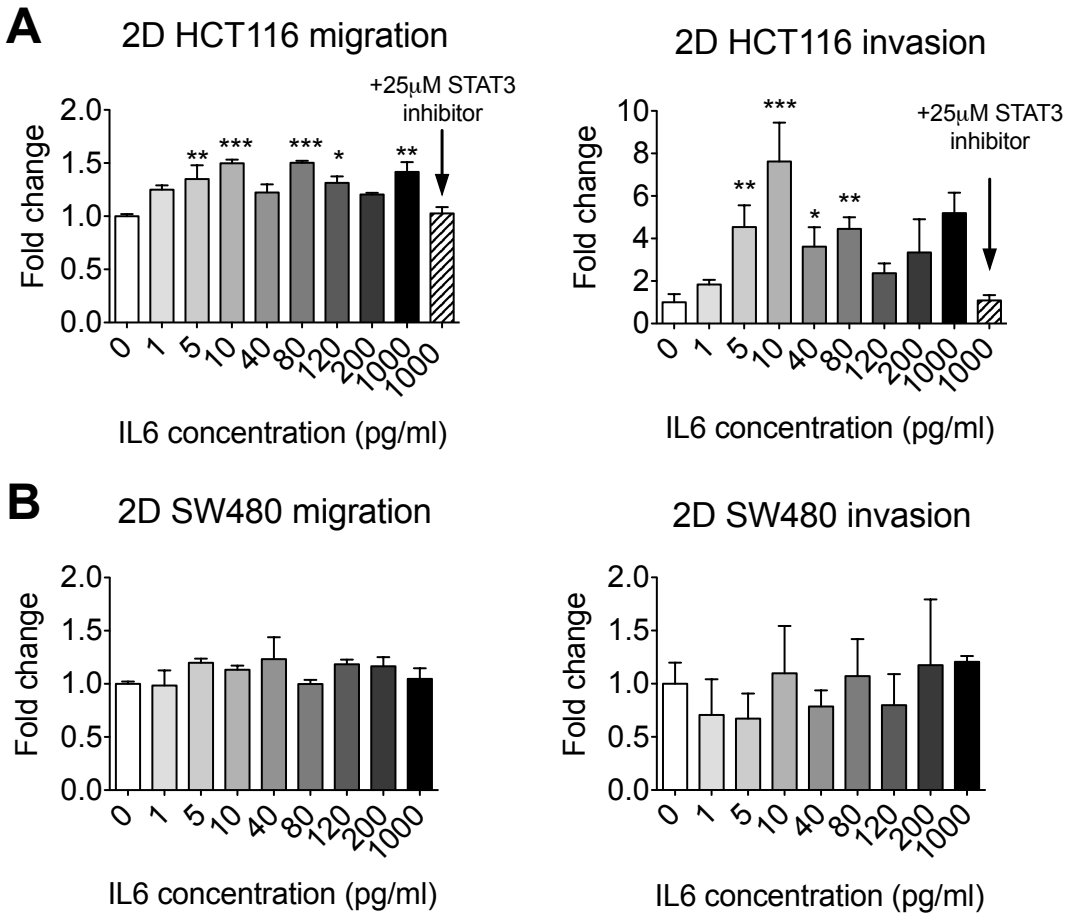

Figure 3.4 Effect of IL6 on HCT116 and SW480 cell migration and invasion. Transwell migration and invasion assays were performed over 72 hours in HCT116 (A) and SW480 (B). Migrated cells to the bottom chamber were quantified using AlamarBlue. Data are expressed as fold change compared to vehicle control (0pg/ml IL6). Significance was calculated using two-way ANOVA with a Bonferroni post-test comparing treated groups to vehicle control (GraphPad Prism 5, $*p<0.05$, $**p<0.01$, $***p<0.001$). Error bars represent the SEM for independent cultures (n=3).

With cells grown as 3D spheroids, the effect observed was not as apparent as in 2D cell culture, however invasion of HCT116 cells was significantly induced with a dose-dependent trend by IL6 treatment while again, no effect was observed in SW480 cells (Figure 3.5). This difference in susceptibility to IL6 treatment between the cell lines is in agreement with the respective responses for cell proliferation.

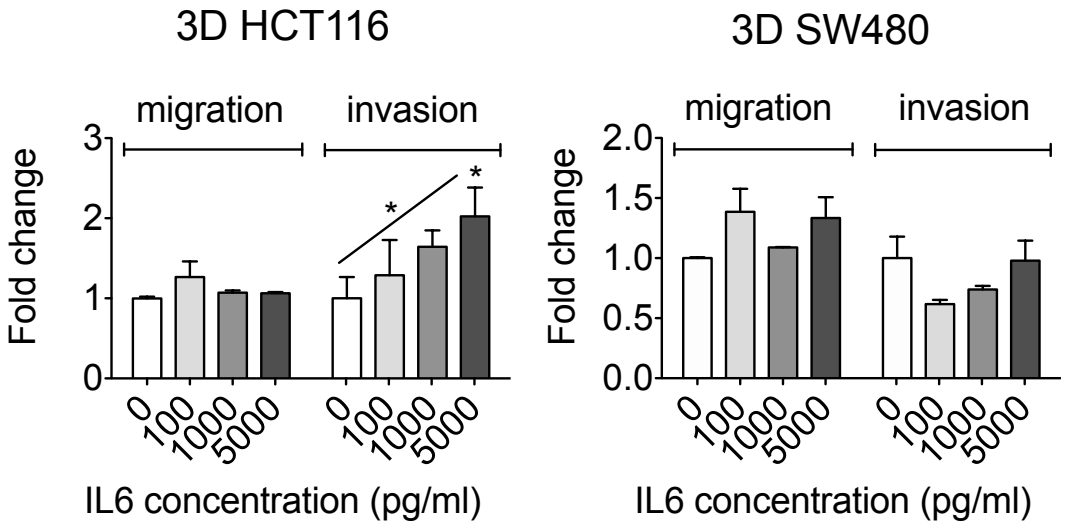

Figure 3.5 Effect of IL6 on migration and invasion of HCT116 and SW480 cells grown as 3D spheroids. Cells were grown on bioscaffolds (Algimatrix) for 10 days. Scaffolds of HCT116 and SW480 cell spheroids were added to the upper chamber and the transwell migration and invasion assay was performed over 72 hours. 10% FBS was added to the bottom chamber as a chemoattractant. For the invasion assay, the upper chamber was coated with matrigel prior to adding the cells. Cells appearing in the bottom compartment were quantified using AlamarBlue. Data are expressed as fold change compared to vehicle control (0pg/ml IL6). Significance was calculated using one-way ANOVA with a Dunnett post-test comparing treated groups to vehicle control and linear trend analysis (GraphPad Prism 5, $*p<0.05$). Error bars represent the SEM for independent cultures (n=3).

Inflammatory cytokines can also act as chemoattractants. To determine whether the effects observed were due to molecular changes within the cells and not a chemoattractive effect of IL6, transwell migration experiments were repeated using IL6 as the chemoattractant in the bottom chamber. No increase in HCT116 migration was noted (Figure 3.6), suggesting that IL6 changes the cellular behaviour of HCT116 cells, potentially through molecular alterations rather than just acting as a chemoattractant.

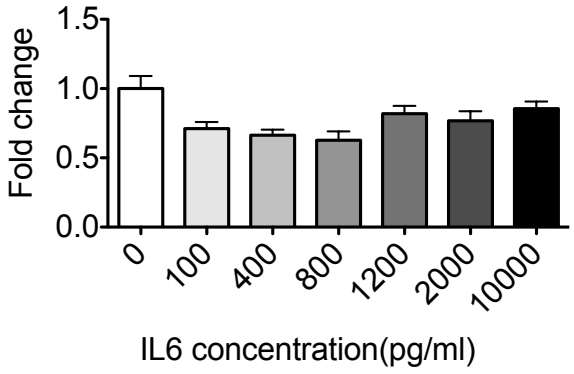

Figure 3.6 Effect of IL6 as a chemoattractant in HCT116 cells. Transwell migration assays was performed over 72 hours with HCT116 cells seeded in the upper chamber. IL6 was added to the bottom chamber as a chemoattractant. Cells appearing in the bottom compartment were quantified using AlamarBlue. Data are expressed as fold change compared to vehicle control (0pg/ml IL6). Significance was calculated using one-way ANOVA with a Dunnett post-test comparing treated groups to vehicle control (GraphPad Prism 5). Error bars represent the SEM for independent cultures (n=3).

In order to gain further insight into these potential IL6-mediated molecular changes, I investigated whether the cells were undergoing phenotypic changes to become more mesenchymal-like.

3.3.2 Does IL6 treatment promote EMT?

Cancer cells commonly undergo phenotypic changes in response to various stimuli. One such change is EMT, where cells lose their epithelial phenotype characterised by tight cell-cell junctions and become mesenchymal-like with the ability to invade surrounding tissue (Lamouille *et al.* 2014). EMT is a key process in metastasis and as I previously observed increased cell migration and invasion in response to IL6, I investigated whether EMT was responsible for this effect. E-cadherin is a cell adhesion molecule expressed at high levels in epithelial cells but lost in mesenchymal cells; its downregulation is a classic marker for EMT. I thus measured *E-cadherin* gene expression in the cells

following IL6 treatment. Interestingly, HCT116 has increased expression of *E-cadherin* while again no significant changes were observed in SW480 and in either cell line when grown as 3D cultures (Figure 3.7), suggesting EMT might not be occurring in response to IL6 in these cells. I therefore investigated whether expression of other genes known to be involved in CRC metastasis were changed in response to IL6.

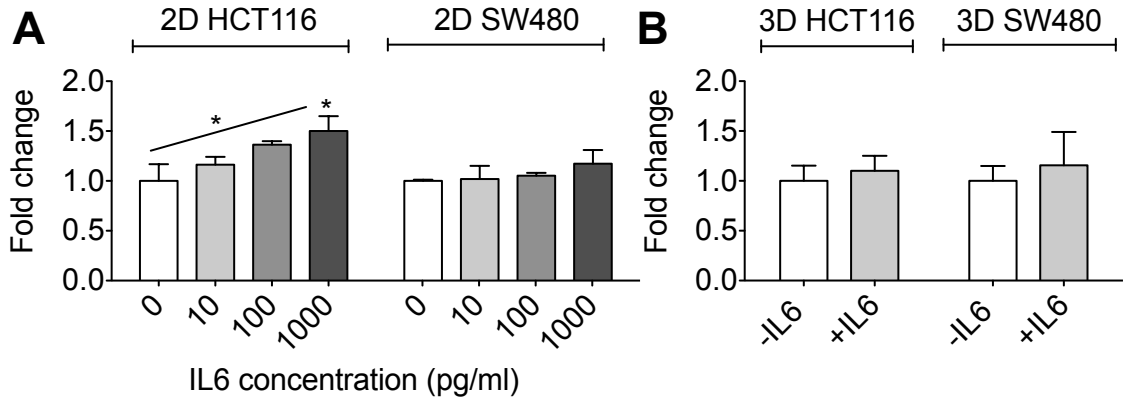

Figure 3.7 IL6 effect on *E-cadherin* gene expression. (A) HCT116 and SW480 cells grown in 2D cell culture were treated with 0, 10, 100 and 1000pg/ml IL6 for 48 and 24 hours respectively. (B) HCT116 and SW480 3D spheroids were grown for 10 days prior to treatment with 1000pg/ml and 5000pg/ml IL6 for 24 hours respectively. *E-cadherin* expression was measured by RT-qPCR. Data were normalised to expression of *GAPDH* and are shown relative to vehicle control. Significance was calculated using Student's *t*-test or one-way ANOVA with a Dunnett post-test comparing treated groups to vehicle control and linear trend analysis (GraphPad Prism 5, $*p<0.05$). Error bars represent the SEM for independent cultures (n=3).

3.3.3 Are oncogenes known to be involved in CRC metastasis regulated by IL6?

A number of genes involved in cancer metastasis and growth were investigated in response to elevated levels of IL6 in CRC cell lines HCT116 and SW480. The tumour-suppressor *p53* expression was not significantly changed in response IL6 treatment (Figure 3.8 and 3.9). Expression of *VEGFA, MMP2, CTSD* and *JAK2*, which are actively involved in different aspects of tumour promotion (as

detailed in the introduction, section 3.1), were upregulated by IL6 in HCT116 cells with a significant dose-dependent trend (Figure 3.8). This effect was not observed in SW480 cells, possibly explaining the lack of cellular response to IL6 treatment observed previously (Chapter 3.3.1). In addition, *JAK2*, *MMP2* and *CTSD* expression appeared to be increased with IL6 treatment in HCT116 3D spheroids (Figure 3.9), however these changes were not statistically significant.

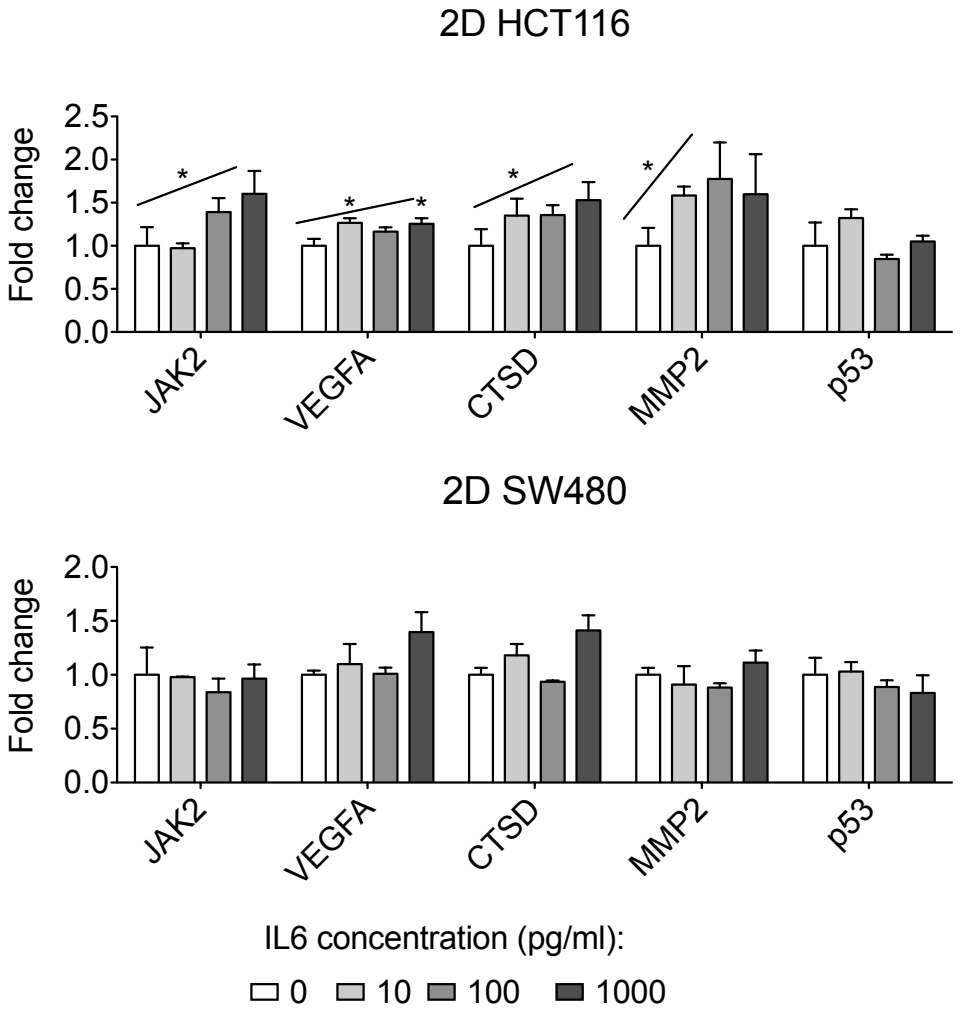

Figure 3.8 IL6 effect on *JAK2, VEGFA, CTSD, MMP2* and *p53* gene expression. HCT116 and SW480 cells were treated with 0, 10, 100 and 1000pg/ml IL6 for 48 and 24 hours respectively. *JAK2, VEGFA, CTSD, MMP2* and *p53* expression were measured by RT-qPCR. Data were normalised to expression of *GAPDH* and are shown relative to vehicle control. Significance was calculated using one-way ANOVA with a Dunnett post-test comparing treated groups to vehicle control and linear trend analysis (GraphPad Prism 5, $^*p<0.05$). Data are presented as a mean of three biological replicates. Error bars represent the SEM for independent cultures.

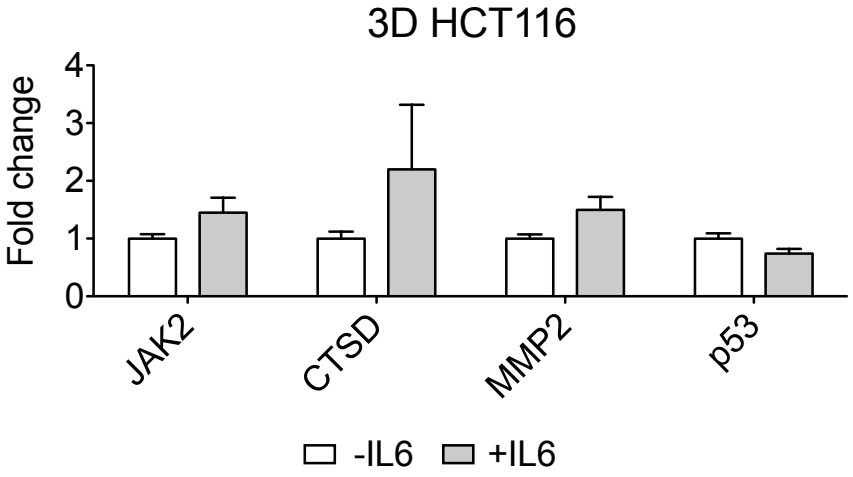

Figure 3.9 *JAK2, CTSD, MMP2* and *p53* gene expression changes in response to IL6 in HCT116 3D spheroids. HCT116 3D spheroids were grown for 10 days prior to treatment with 0 and 1000pg/ml IL6 for 24 hours. *JAK2, CTSD MMP2* and *p53* expression were measured by RT-qPCR. Data were normalised to expression of *GAPDH* and are shown relative to vehicle control. Significance was assessed using a Student's *t*-test (GraphPad Prism 5). Data are presented as a mean of three biological replicates. Error bars represent the SEM for independent cultures.

As I previously observed that STAT3 inhibition prevented the IL6-mediated induction of cell proliferation, I investigated the effect of STAT3 inhibition on *JAK2, CTSD* and *MMP2* gene expression in HCT116 cells, as *VEGFA* is already known to be regulated by STAT3 (Wei *et al.* 2003). Co-treatment with IL6 and the STAT3 inhibitor appeared to bring expression levels back to control values, however none of these changes were significant; thus STAT3 may be involved in regulating expression of these genes but further validation is required (Figure 3.10).

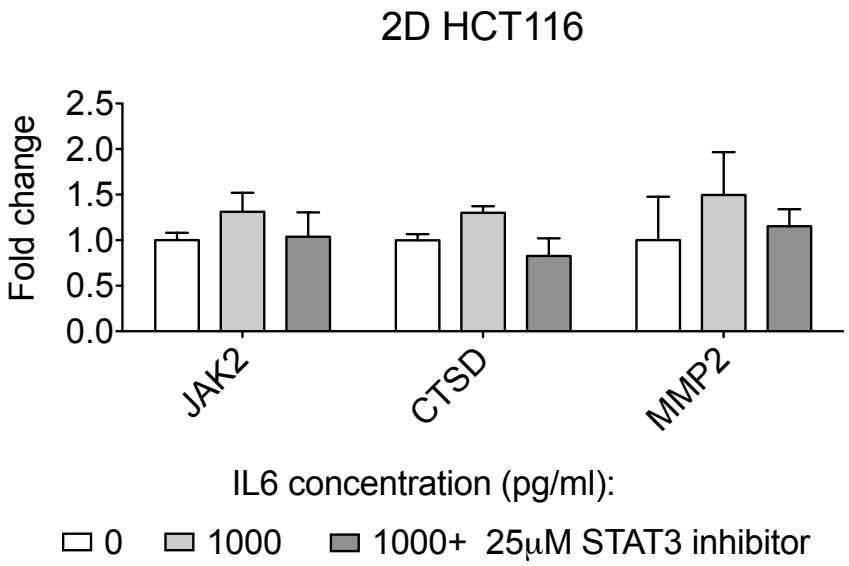

Figure 3.10 STAT3 involvement in *JAK2, CTSD* and *MMP2* gene expression. HCT116 cells were treated with 0, 1000pg/ml IL6 and IL6 + 25μM STAT3 inhibitor for 48 hours. *JAK2, CTSD* and *MMP2* expression were measured by RT-qPCR. Data were normalised to expression of *GAPDH* and are shown relative to vehicle control. Significance was calculated using one-way ANOVA with a Dunnett post-test comparing treated groups to vehicle control (GraphPad Prism 5). Data are presented as a mean of three biological replicates. Error bars represent the SEM for independent cultures.

3.3.4 Can IL6 treatment induce changes in miRNA expression?

I tested expression of a number of CRC tumour-suppressing (miR375, miR124a and let7a) and tumour-promoting miRNAs (miR31, miR21, miR135b and miR96). MiR29b expression was also investigated due to its controversial role in tumourigenesis; indeed it has been found to have oncogenic (Wang *et al.* 2011; Xu *et al.* 2013) and tumour-suppressive roles (Jia *et al.* 2014; Melo & Kalluri 2013; Subramanian *et al.* 2014; Wang *et al.* 2014; Inoue *et al.* 2014). In HCT116 grown in 2D culture, miR135b, miR21 and miR29b appear to be upregulated in response to IL6 with significant dose-dependent trends, while only miR21 and miR29b were upregulated in SW480 cells (Figure 3.11). In 3D cell culture, no significant changes were observed in miRNA expression with IL6 treatment (Figure 3.12).

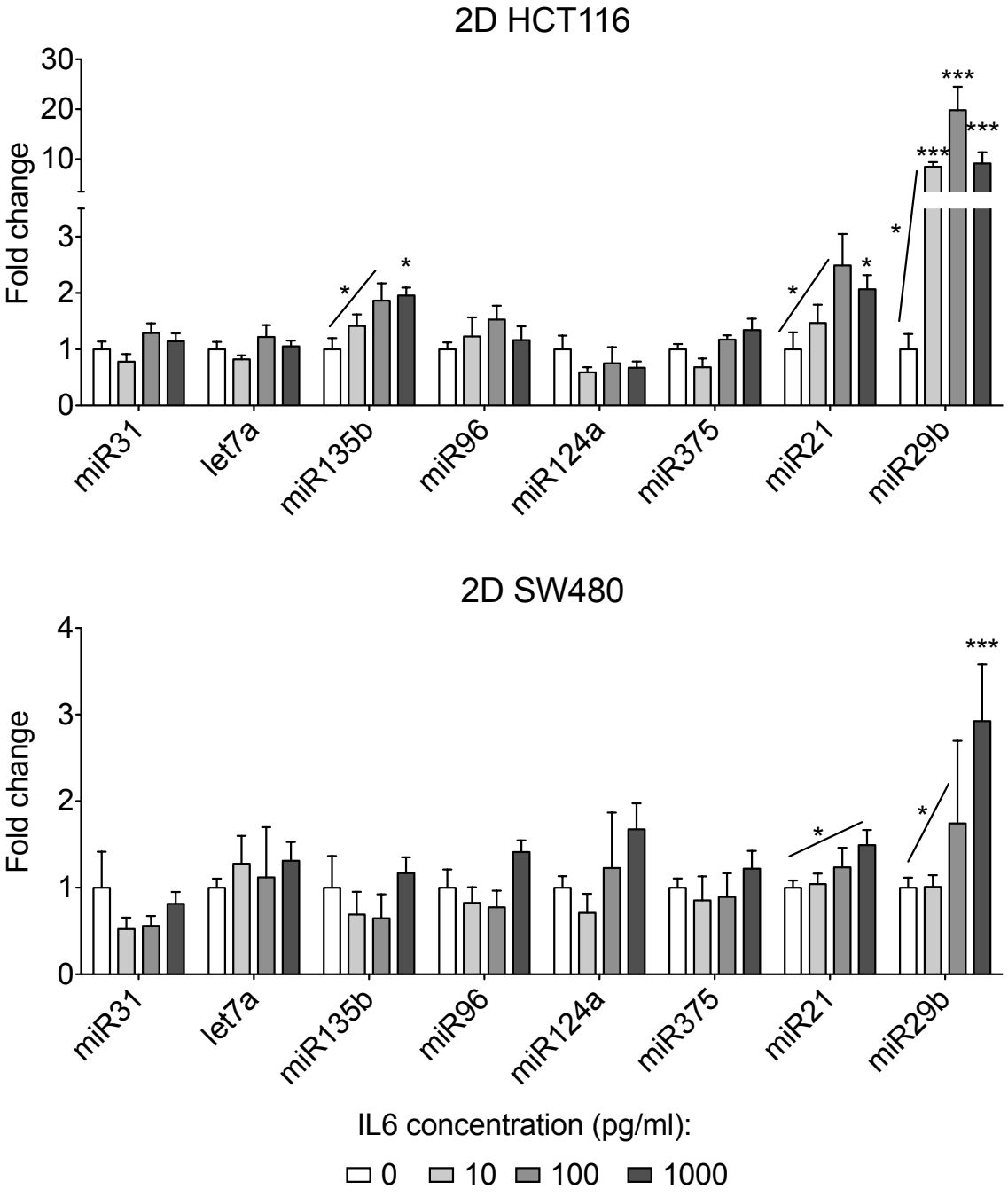

Figure 3.11 IL6 effect on miRNA expression. HCT116 and SW480 cells grown as monolayers, were treated with 0, 10, 100 and 1000pg/ml IL6 for 48 and 24 hours respectively. MiRNA expression was measured by RT-qPCR. Data were normalised to expression of U6 RNA and are shown relative to vehicle control. Significance was calculated using one-way ANOVA with a Dunnett post-test comparing treated groups to vehicle control and linear trend analysis (GraphPad Prism 5, *p<0.05, ***p<0.001). Data are presented as a mean of three biological replicates. Error bars represent the SEM for independent cultures

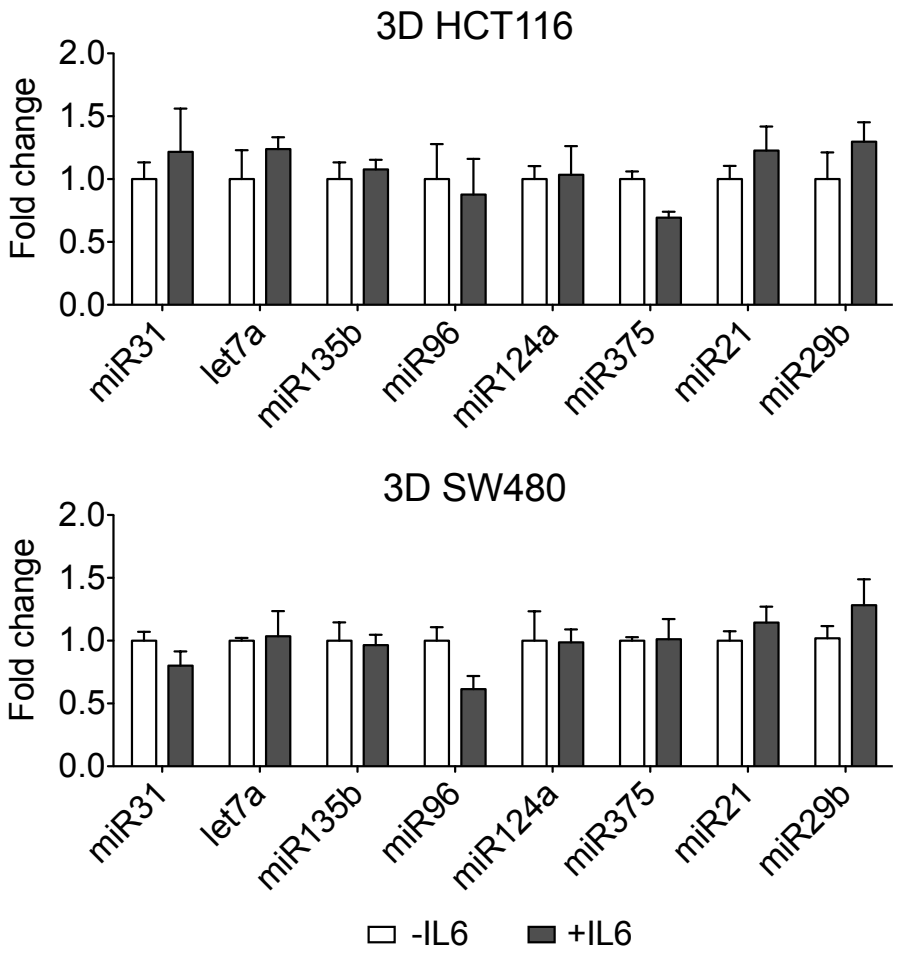

Figure 3.12 MiRNA expression changes in response to IL6 in HCT116 and SW480 3D spheroids. HCT116 and SW480 3D spheroids were grown for 10 days prior to treatment with 1000pg/ml and 5000pg/ml IL6 for 24 hours respectively. MiRNA expression was measured by RT-qPCR. Data were normalised to expression of U6 RNA and are shown relative to vehicle control. Significance was assessed using Student's *t*-test (GraphPad Prism 5). Data are presented as a mean of three biological replicates. Error bars represent the SEM for independent cultures.

3.3.5 What effects do miR21 and miR29b have on CRC cells?

Following the observation that IL6 induces miR21 and miR29b expression *in vitro*, I determined the effect of these miRNAs on cell growth, migration and invasion using overexpression studies (Figure 3.13 A). I found that both miRNAs appeared to induce cell proliferation however these changes were not significant compared to the mimic control (Figure 3.13 B). Additionally, miR29b appeared to induce cell invasion in the HCT116 cell line (Figure 3.13 C), suggesting a potential pro-metastatic role for miR29b. As observed previously (section 3.3.1), induction of invasion was not observed in SW480. In order to determine whether elevated levels of IL6 can induce miRNA expression changes *in vivo,* I tested human tumour and adjacent normal samples for IL6 and miRNA expression.

3.3.6 Do IL6 and miRNA expression change in malignant colorectal tissue?

Tumour tissue and adjacent normal samples resected from CRC patients were analysed for IL6, miR21 and miR29b expression. However, IL6 expression was not significantly increased in the tumour tissue compared to adjacent normal in this sample set (Figure 3.14 A and B). Additionally, no change in miR29b or miR21 expression was observed in these samples (Figure 3.14 C and D). Therefore, these data do not exclude the possibility that IL6 may regulate miR29b and miR21 *in vivo*, however this remains unverified in the current study. Furthermore, the sample set only included 7 patients, which is too limited a number to accurately assess potential effects.

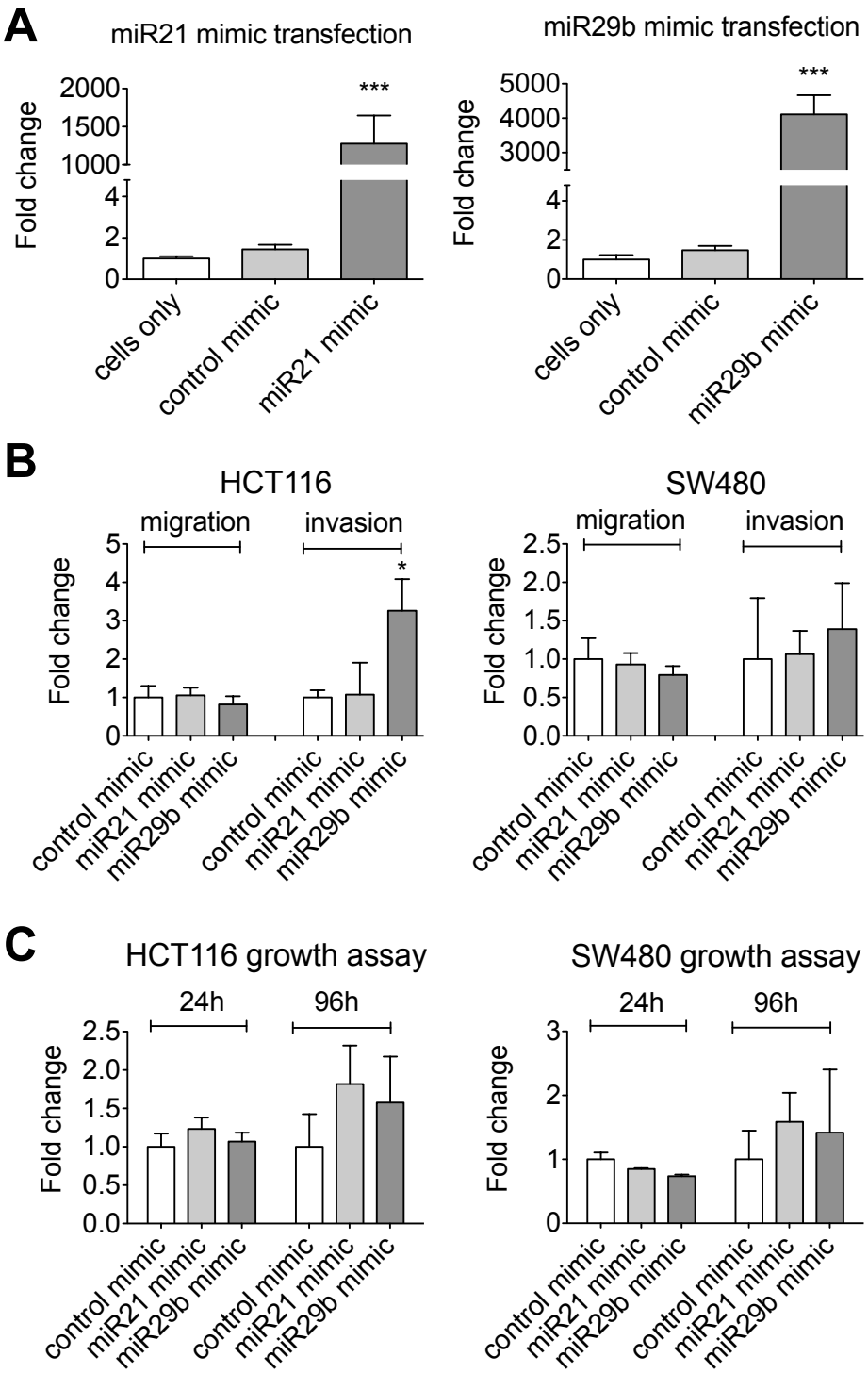

Figure 3.13 Effect of miR29b and miR21 expression on CRC cells. (A) miR21 and miR29b mimics were transfected into the cells. MiRNA expression data were normalised to expression of U6. (B) Transwell migration and invasion assays were performed over 72 hours. Migrated cells were quantified using AlamarBlue. (C) Cell proliferation was determined using AlamarBlue. Data are shown relative to mimic control. Significant differences were calculated using one-way ANOVA followed by a Dunnet post-test (GraphPad Prism 5, ***p<0.001, *p<0.05). Error bars represent the SEM for independent cultures (n=3).

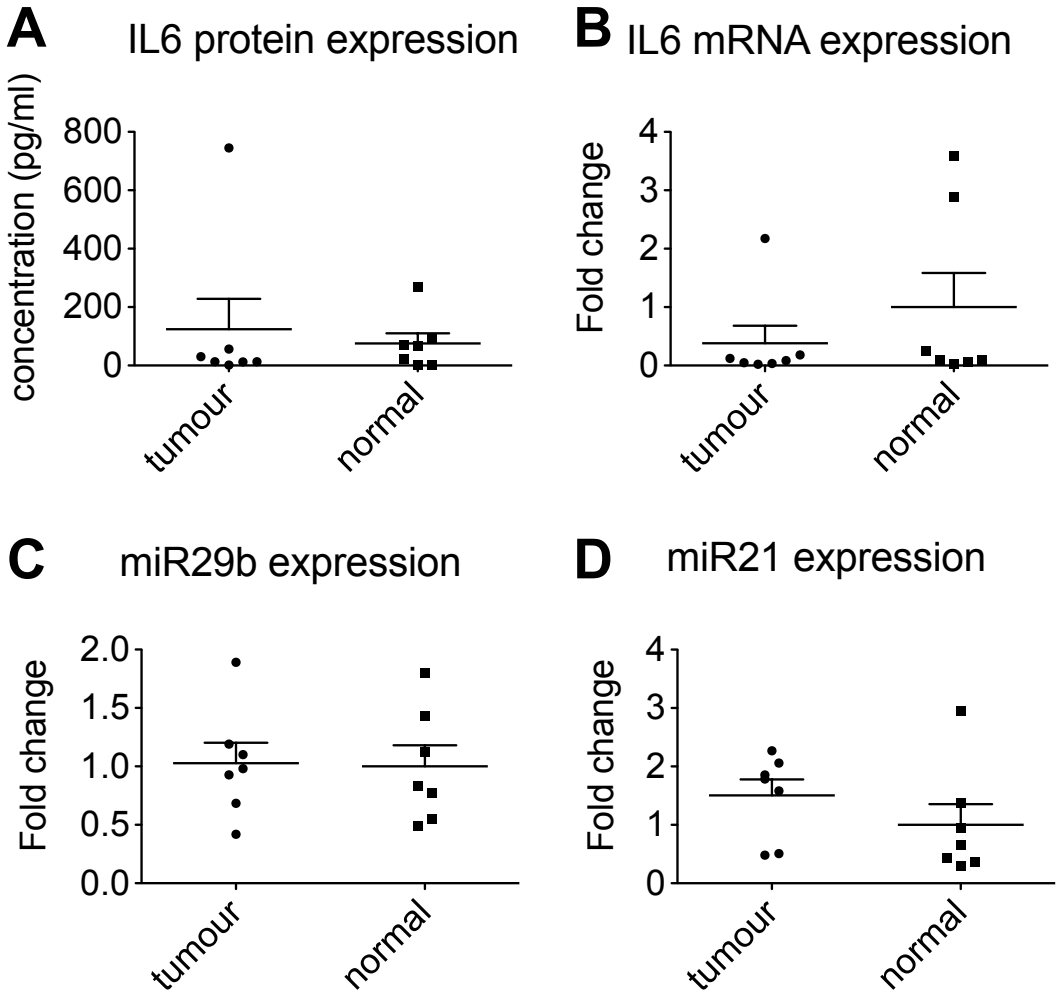

Figure 3.14 IL6, miR29b and miR21 expression in CRC tissue samples. IL6 concentration was measured in protein extracted from tissue samples (A). IL6 mRNA (B), miR29b (C) and miR21 (D) expression were measured by RT-qPCR. Gene expression data were normalised to expression of *GAPDH* and U6 for mRNA and miRNA expression respectively, and are shown relative to normal tissue control. Significant differences between tumour and normal tissue were calculated using a Mann-Whitney test (GraphPad Prism 5, n=7).

3.4 Discussion

IL6 is a key regulator of the inflammatory response; it is also thought to be important in CRC initiation and progression, and increased levels of IL6 are present in CRC tumours and stroma (Maihöfner *et al.* 2003; Nagasaki *et al.* 2014; Chung *et al.* 2006; Lu *et al.* 2015; Uchiyama *et al.* 2012). In the current study, I investigated the effect of IL6 on CRC cell behaviour *in vitro* using HCT116 and SW480 cells grown as 2D and 3D cultures. I established that elevated levels of IL6 promote CRC cell proliferation, migration and invasion, which are key behavioural features of metastatic cells. These changes were accompanied by increased expression of oncogenes such as *JAK2*, *MMP2*, *CTSD* and *VEGFA*. STAT3 signalling inhibition appeared to reverse this effect, although the IL6-induced changes were not significant and thus the effect of STAT3 could not be confirmed with statistical significance. Again, the lack of STAT3 inhibitor only control means any non-specific effect of the inhibitor is not accounted for in these experiments (more detail on the implication of this in Chapter 2, section 2.4). Importantly, the doses of IL6 used in these studies were in-keeping with IL6 levels found in the TME (Nagasaki *et al.* 2014) and under these conditions, induced changes may be subtle and difficult to confirm statistically with small experimental numbers. Further experimentation using higher IL6 doses is needed to validate any potential changes observed here.

Furthermore, the changes observed in response to IL6 were primarily observed in HCT116 cells compared to SW480, possibly due to the fact that no changes in *MMP2* and *CTSD* expression were observed in the latter. When comparing relative expression of these genes in both cell lines, I observed that these are expressed at much higher levels in SW480 cells compared to HCT116 (Figure

3.15), thus possibly explaining their reduced susceptibility to induction by IL6.

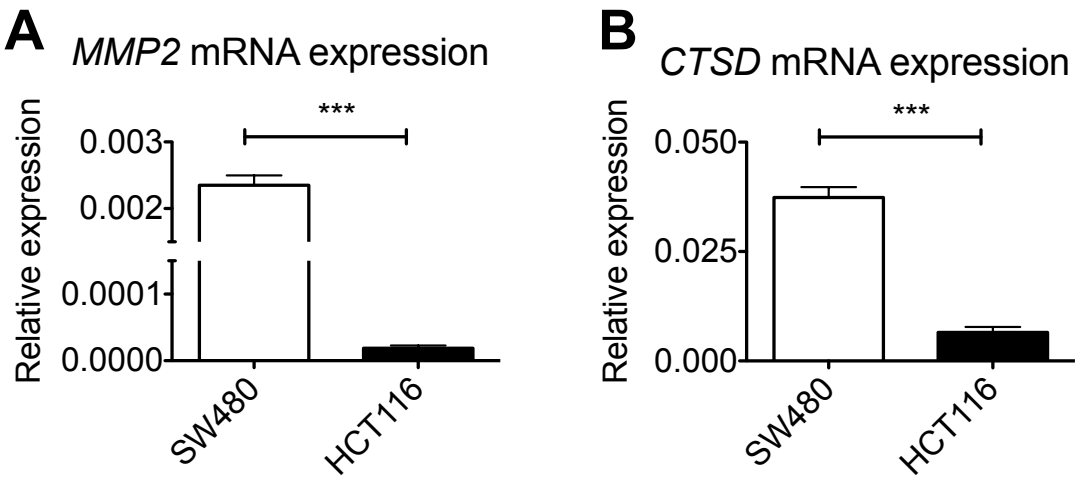

Figure 3.15 *CTSD* and *MMP2* gene expression in HCT116 and SW480. *MMP2* (A) and *CTSD* (B) expression were measured by RT-qPCR in HCT116 and SW480 cells. Data are shown relative to expression of *GAPDH*. Significance was assessed using Student's *t*-test (GraphPad Prism 5, ***$p<0.001$). Data are presented as a mean of three biological replicates. Error bars represent the SEM for independent cultures.

Furthermore, only a select few genetic markers of cell migration and invasion were investigated here. Such a targeted approach can miss key changes mediated by IL6 and therefore a more complete study should include an investigation of the IL6 effect on global gene regulation. Of these select few gene targets, knockdown and overexpression studies could be useful additional studies to establish their involvement in the behavioural changes observed following IL6 treatment.

Also, HCT116 are known to have cancer stem cell (CSC)-like properties (Botchkina *et al.* 2009); CSC cells are thought to be important drivers of cancer progression playing a key role in metastasis and drug-resistance. As with normal stem cells, these cells are regulated by their microenvironment and the IL6-STAT3 pathway has been reported to be an important regulator of CSCs

(Liu *et al.* 2014; Zhu *et al.* 2014; Korkaya *et al.* 2011), providing another plausible explanation for the higher susceptibility of HCT116 cells to IL6 treatment compared to SW480 cells.

I also observed changes in miRNA expression in response to IL6, in particular increased expression of miR21 and miR29b. MiR21 is a known oncogenic miRNA as it targets pro-apoptotic *PTEN* and *PDCD4* expression (Asangani *et al.* 2008; Liu *et al.* 2012; Li *et al.* 2014). MiR21 overexpression has previously been reported in CRC tissue and it is also thought to be associated with metastasis. It is known to be regulated by STAT3 (Yang *et al.* 2010), which is activated by IL6, thus my data concur with previous observations. Mir29b has a disputed role in cancer; it has been reported to be both tumour promoting and suppressing (Wang *et al.* 2014; Luo *et al.* 2011; Slaby *et al.* 2009; Jiang *et al.* 2014). I found that miR29b induced cell invasion, suggesting a potential pro-tumourigenic role in the current CRC model. MiR21 and miR29b have also been reported as circulatory miRNAs able to integrate into surrounding cells acting as paracrine signalling molecules (Ogata-Kawata *et al.* 2014; Dorval *et al.* 2013; Guay & Regazzi 2013; Yang *et al.* 2012; Wu *et al.* 2010; Valadi *et al.* 2007), suggesting these miRNAs may play a role in the TME in addition to their potential oncogenic roles within the CRC cells.

In addition, it appeared that IL6 effects observed in cells cultured as 3D spheroids were generally less obvious than in cells grown as 2D cultures. Decreased drug sensitivity has been reported when comparing cells cultured in 3D to 2D (Li *et al.* 2008; Doillon *et al.* 2004), and these differences are likely due to the changes in tissue architecture: access to the inner cells of the spheroid is limited resulting in uneven treatment exposure levels. While this

effect was only mildly observed in the previous chapter (Chapter 2), it appears to be more apparent with the current study, particularly when no increase in miR29b and miR21 were observed in 3D cultured cells in response to IL6 compared to an induction in 2D cells of up to 20-fold (Figure 3.11). Uneven exposure of cells to treatment would be an unlikely explanation for such differences; other possible reasons could include differences in miRNA processing mechanisms or higher background expression of these miRNAs in 3D cultures compared to 2D cultures. However, when comparing expression levels in untreated cells (Appendix C, Figure S.11), miR21 and miR29b levels appear to be similar between cells grown in 2D and 3D cultures. Another possibility is that 3D cultures are more effective at secreting these miRNAs, and thus higher levels of the miRNAs in response to IL6 are readily transported out of the cells making them undetectable when analysing miRNA expression within the cells. This could be verified by analysing miRNA levels in the cell culture medium of 3D spheroids treated with IL6 and comparing these to that of 2D cultures. The lack of response in 3D cultured cells compared to the observed 2D culture findings is not readily explainable; therefore it is important to understand why these differences occur and which system more closely relates to *in vivo* models and relevance to the CRC disease state.

As an attempt to determine *in vivo* relevance of the current *in vitro* findings, I investigated possible changes in IL6, miR21 and miR29b expression in CRC tumour tissue compared to adjacent normal. In the current sample set no significant changes were observed. However, studies with larger CRC tissue sample sets have reported that IL6, miR21 and miR29b expression are increased in malignant cells (Maihöfner *et al.* 2003; Xu *et al.* 2012; Tan *et al.*

2013; Uchiyama *et al.* 2012; Nagasaki *et al.* 2014; Lu *et al.* 2015); therefore regulation of these miRNAs by IL6 may still occur *in vivo* but the data presented here were insufficient to draw any conclusions. In addition, using adjacent normal tissue from the same patients as a control cohort may not be suitable as it has been suggested that these tissues may be undergoing phenotypic changes due to their proximity to the tumour cells and microenvironment (Clare *et al.* 2012). It is also important to note that only a very small number of patients (n=7) were used in the current study, which is not a large enough population to provide an accurate representation of the CRC cohort. Given these limitations, the significance of these results is difficult to assess and a larger study is necessary in order to draw any conclusions.

Interestingly, it was recently discovered that miR21 and miR29a are able to bind Toll-Like receptor 8 (TLR8) contained within endosomes in immune cells to induce an inflammatory response (Fabbri *et al.* 2012). Given that I have observed IL6-medieated increased expression of miR21 and miR29b, a miRNA closely related to miR29a, I investigated the potential role of IL6 and these miRNAs in promoting crosstalk between tumour cells and immune cells in the microenvironment, which I have discussed in the next chapter (Chapter 4).

3.5 Summary

Taken together, the data from this study demonstrated that IL6 alters CRC cell behaviour accompanied by changes in gene and miRNA expression (Figure 3.16). MiR21 and miR29b were both upregulated by IL6 treatment in 2D culture and thus have emerged as interesting miRNAs due to their reported circulatory nature. Therefore, the next chapter will concentrate on their role in the TME using an *in vitro* co-culture system.

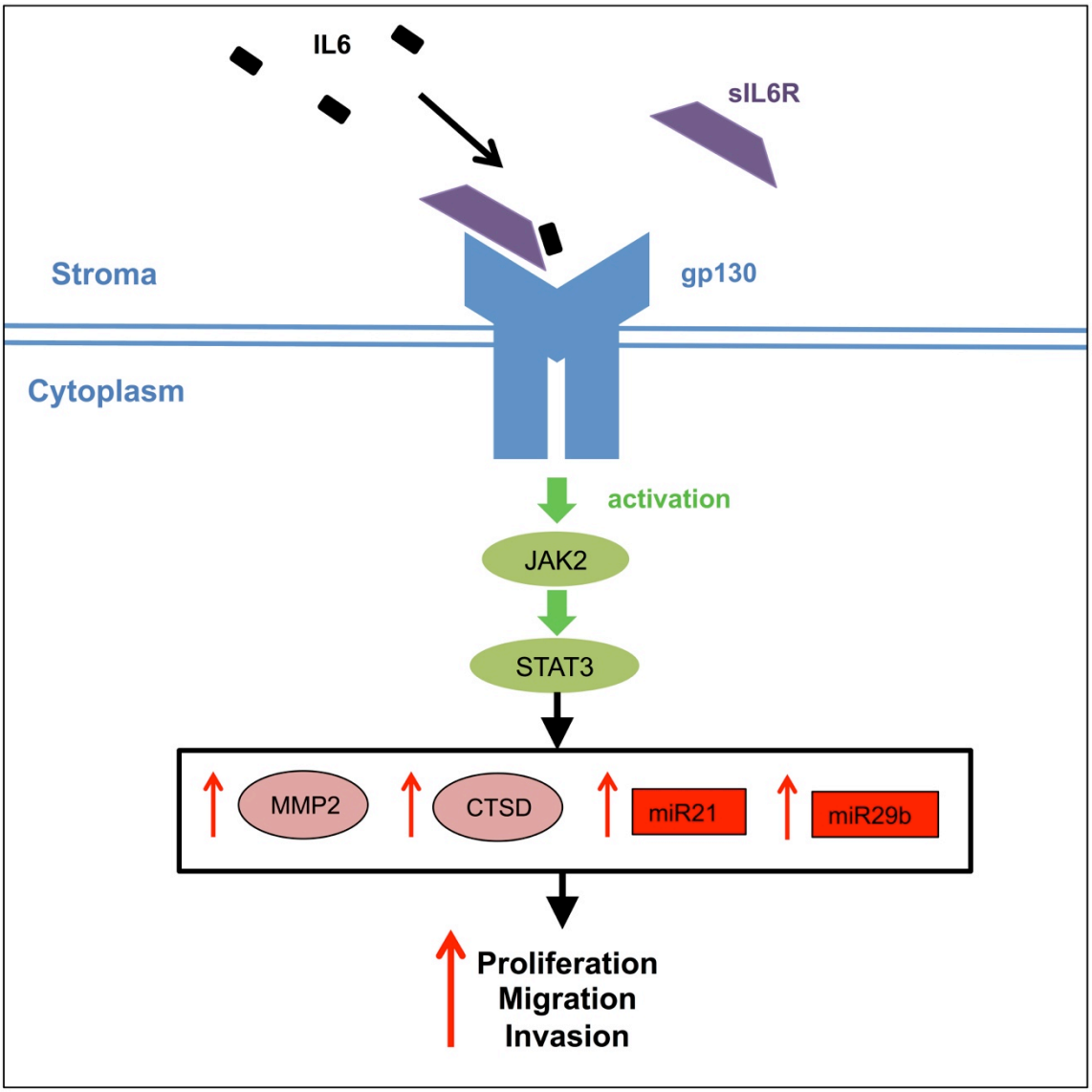

Figure 3.16 Suggested model for IL6-mediated CRC cell behavioural changes through altered gene and microRNA expression. IL6 activates STAT3 to induce expression of *MMP2*, *CTSD*, miR21 and miR29b resulting in increased cell proliferation, invasion and migration.

- Chapter 4 -

Interleukin-6 promotes intercellular communication in the tumour microenvironment *via* miR21 and miR29b.

N.B. Results from this chapter have been included in the following peer-reviewed publication:

Patel, S.A.A. and Gooderham, N.J., 2015. Interleukin-6 mediates crosstalk between immune and cancer cells via miR21 and miR29b. Molecular Cancer Research (In Press).

4.1 Introduction

Roughly half the cells contained in a malignant tumour are non-cancerous cells. Indeed, tumour cells are surrounded and infiltrated by a variety of stromal cells including fibroblasts and vascular endothelial cells, as well as immune cells, which interact with malignant cells to create the TME (Balkwill *et al.* 2012; Bhome *et al.* 2015).

This complex environment is thought to promote cancer progression and has been identified as a potential target for therapy (Albini & Sporn 2007; Balkwill & Mantovani 2012; Noy & Pollard 2014). Understanding cell-cell communication within the TME is key to this therapeutic approach. The tumour cells are thought to communicate *via* extracellular signals with surrounding cells to promote their progression, and cytokines have emerged as important mediators of this crosstalk (detailed in Chapter 1, section 1.3).

IL6 is secreted by tumour cells as well as surrounding CAFs and immune cells resulting in presence of high levels in the TME (stromal cells can secrete up to 8000pg/ml; Nagasaki *et al.*, 2014). Importantly, IL6 is also thought to play an important role in cancer progression (Nagasaki *et al.* 2014; Waldner *et al.* 2012; Taniguchi & Karin 2014; Grivennikov *et al.* 2009). In the previous chapter, I have demonstrated that IL6 can alter behaviour of CRC cells and induce expression of miRNAs miR21 and miR29b.

In recent years, a new role for miRNAs as paracrine signalling molecules has emerged (Valadi *et al.* 2007). This hormone-like function is particularly apparent in the immune response where miRNAs facilitate the crosstalk between different types of immune cells (Mittelbrunn *et al.* 2011) and can also activate human immune cells by binding to TLR8 (Fabbri *et al.* 2012). Indeed,

miR21 and miR29a can act as TLR8 ligands in a similar way to viral single-stranded RNA, resulting in stimulation of the host immune cell to produce and secrete pro-inflammatory cytokines including IL6.

In this chapter, I have investigated the potential role of IL6, miR21 and miR29b in mediating intercellular communication between CRC cells and adjacent immune cells using an *in vitro* co-culture model.

4.2 Materials and Methods

4.2.1 Cell culture

The human monocyte-like THP1 cell line was kindly gifted by Dr James Pease (Imperial College London, UK) and routinely cultured in RPMI1640 medium (GIBCO, Life technologies, Paisley, UK) supplemented with 10% FBS, 100units/ml penicillin, 100μg/ml streptomycin and 2mM *L*-glutamine (GIBCO, Life technologies). HCT116 and SW480 were cultured as 2D and 3D cultures as previously described (section 2.2.1). For co-culture experiments, THP1 suspension cells were added to the CRC adherent cell lines. All cells were incubated at 37°C in a humidified incubator (5% CO_2).

4.2.2 IL6 and lipopolysaccharide treatment

HCT116 and SW80 cells were treated as previously described (section 2.2.2). For THP1 cells, 1μg/ml lipopolysaccharide (LPS) purified from *Escherichia coli* 0127:B8 by phenol extraction (Sigma-Aldrich, Dorset, UK) and dissolved in cell culture medium was added to the cells for 3 hours to in order to stimulate pro-inflammatory cytokine secretion.

4.2.3 Transwell migration and invasion assays

Assays were performed as previously described (section 3.2.4).

4.2.4 RNA extraction

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Life technologies) as previously described (section 2.2.4). For miRNA isolation from cell culture medium, miRVANA PARIS kits were used (Applied biosystems, Life technologies) with 400μl of conditioned medium according to the manufacturer's protocol. Synthetic *C. elegans* miR39 was spiked in to every

sample at a concentration of 0.15 fmol (Ambion. Life technologies) and used to normalise miRNA expression in culture medium.

4.2.5 Reverse transcription and qPCR

Reverse transcription and qPCR were performed as previously described (section 2.2.5). Details of primers used are included in Appendix A.

4.2.6 Transfection of miRNA mimics

Transfection experiments were performed as previously described (section 3.2.8) using THP1 cells

4.2.7 Enzyme-linked immunosorbent assay (ELISA)

IL6 was quantified in cell culture medium using the Human IL6 Quantikine ELISA Kit according to manufacturer's protocol (R&D systems, Abingdon, UK). The kit was kindly gifted by Dr Marc Dumas (Imperial College London, UK). Absorbance was measured in a Synergy H1 plate reader (Biotek, Potton, UK).

4.2.8 Statistical Analysis

Statistical significance was assessed as previously described (section 2.2.10).

4.3 Results

4.3.1 Can conditioned medium from immune cells have an effect on CRC cell behaviour?

In the TME, immune cells interact with cancer cells *via* a complex mixture of cytokines including IL6. To recapitulate this environment, conditioned medium from activated THP1 human monocytic cells containing an array of pro-inflammatory cytokines including IL6, IL1β and TNFα, was added to CRC cells. THP1 cells were first stimulated for 3 hours with 1μg/ml LPS and IL6 expression and secretion levels were measured (Figure 4.1).

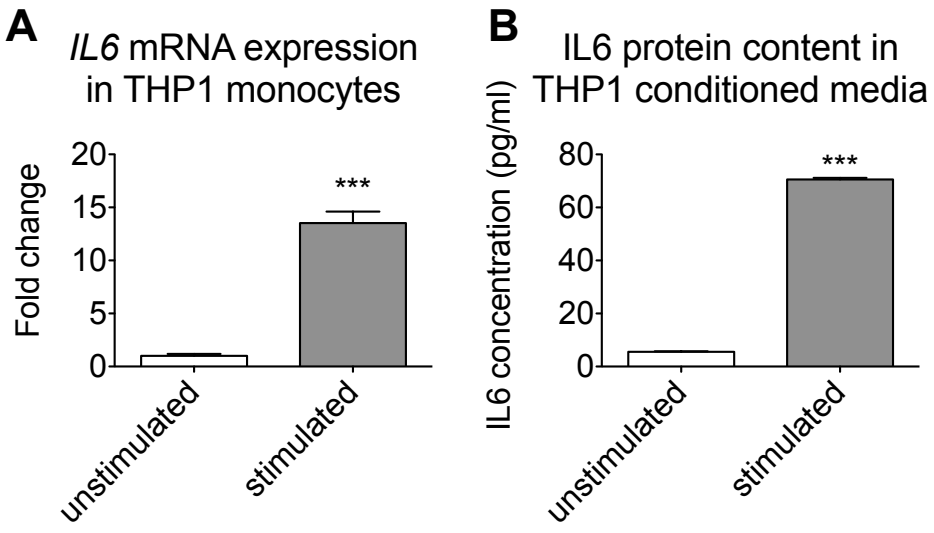

Figure 4.1 IL6 expression and secretion in LPS-stimulated THP1 cells. THP1 cells were treated with 1μg/ml LPS for 3h. (A) IL6 mRNA expression in THP1 cells was measured by RT-qPCR. Data were normalised to expression of *GAPDH* and are shown relative to vehicle control. (B) IL6 secretion was measure by ELISA assay in conditioned media from triplicate cultures of THP1 cells. Data are expressed as IL6 concentration in pg/ml. Significance was assessed using Student's *t*-test (GraphPad Prism 5, ***$p<0.001$). Error bars represent the SEM (n=3).

Conditioned medium from the stimulated THP1 cells was then used to treat 2D HCT116 and SW480 cells for 24 hours. A transwell assay was performed to determine the effect of the conditioned medium on cell migration and invasion (Figure 4.2). As with IL6 treatment (Chapter 3, section 3.3.1), a significant 4 fold increase in cell invasion was observed in HCT116 cells treated with conditioned medium from LPS stimulated THP1 cells. This induction of invasion is similar to that seen with 80pg/ml of IL6 alone (Chapter 3, section 3.3.1), suggesting that this effect is primarily mediated by IL6 and not the other pro-inflammatory cytokines present in the conditioned medium. However, cell migration was not induced and no significant changes were noted in SW480 cells and HCT116 grown as 3D spheroids, which is similar to our findings with IL6 treatment alone (Chapter 3, section 3.3.1). While MMP2 expression appeared to be increased in HCT116 cells following a 24 hour treatment with conditioned medium, these changes were not significant (Figure 4.3).

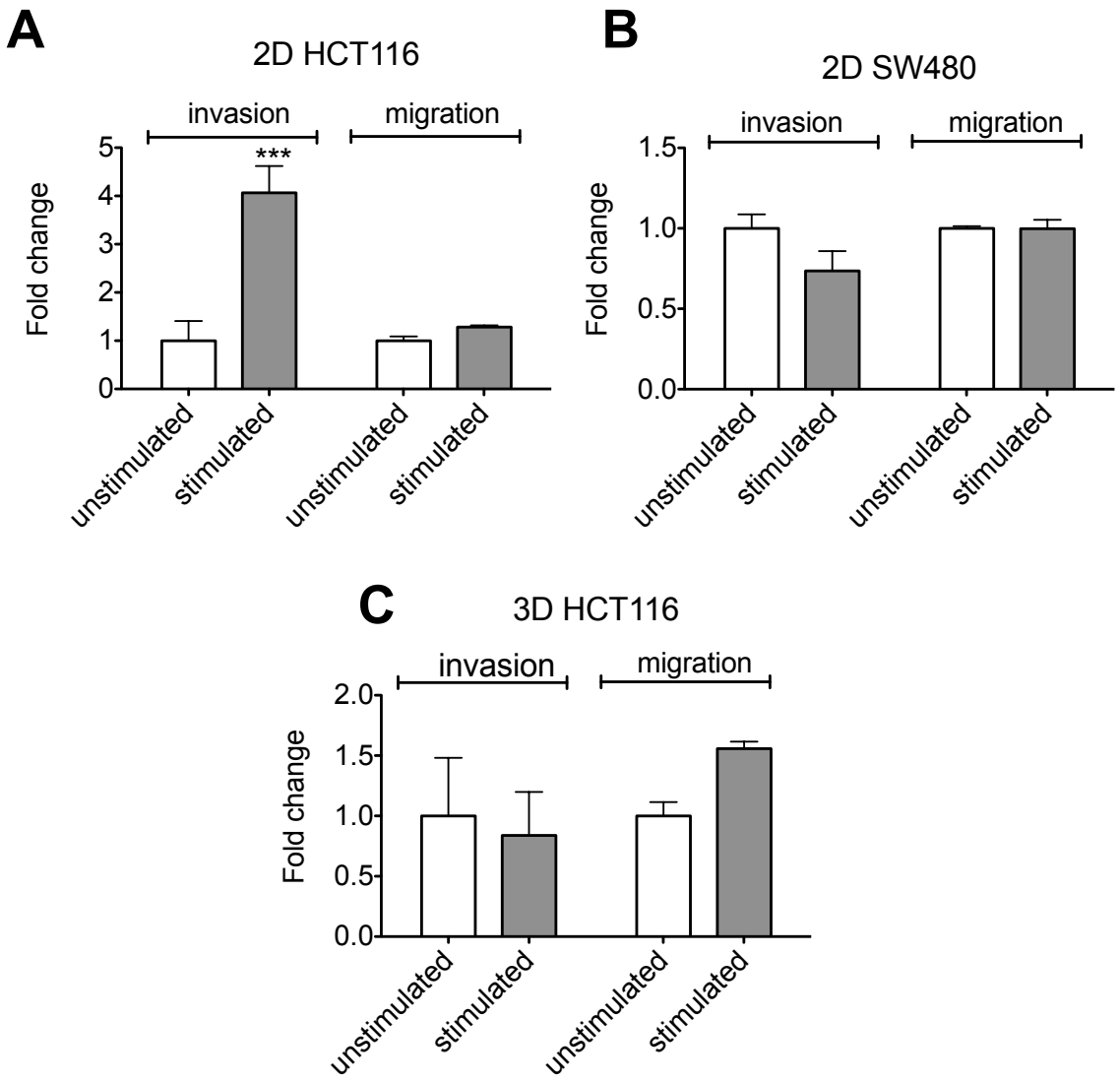

Figure 4.2 Effect of LPS-stimulated THP1 culture medium on SW480 and HCT116 cell migration and invasion. THP1 cells were treated with 1μg/ml LPS for 3 hours, conditioned medium was collected and used to treat SW480 and HCT116 cells for a further 24 hours. A transwell migration and invasion assay was performed over 72 hours with HCT116 (A), SW480 (B) cells grown in 2D culture and 3D HCT116 spheroids (C). Cells were added to the upper chamber and 10% FBS was added to the bottom layer as a chemoattractant. For the invasion assay, the upper chamber was coated with matrigel prior to cell seeding. Cells in the bottom compartment were quantified using AlamarBlue. Data are expressed as fold change of vehicle control (non-stimulated THP1 conditioned medium). Significance was assessed using Student's *t*-test (GraphPad Prism 5, ***p<0.001). Error bars represent the SEM for independent cultures (n=3).

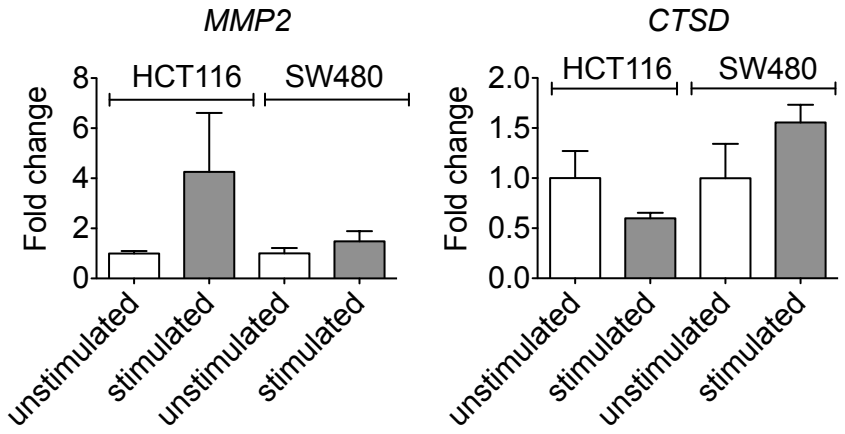

Figure 4.3 Effect of activated THP1 culture medium on *MMP2* and *CTSD* expression in CRC cells. THP1 cells were treated with 1μg/ml LPS for 3 hours. Conditioned medium was used to treat SW480 and HCT116 cells for 24 hours. *CTSD* and *MMP2* expression were measured by RT-qPCR. Data were normalised to expression of *GAPDH* and are shown as a fold change of vehicle control (non-stimulated THP1 conditioned medium). Significance was assessed using Student's *t*-test (GraphPad Prism 5). Error bars represent the SEM for independent cultures (n=3).

4.3.2 Do CRC cells have an effect on surrounding immune cells?

In the current model, only THP1 cells are able to secrete IL6, as HCT116 and SW480 do not produce detectable amounts of *IL6* mRNA (Appendix A, Figure S.4). So as to determine whether the cancer cells can stimulate THP1 cells to sustain IL6 production, I cultured HCT116 or SW480 cells with THP1 cells for 24 hours and measured IL6 expression and secretion by the THP1 cells (Figure 4.4). Interestingly, co-culture alone was not able to significantly induce IL6 production by THP1 cells. However, when the CRC cells were pre-treated with IL6 for 24 hours prior to co-culture, IL6 production was significantly induced in the THP1 cells suggesting that factors released by IL6-treated CRC cells but not untreated CRC cells are responsible for THP1-mediated IL6 secretion. Interestingly, co-culture with IL6-treated SW480 cells was more effective at inducing IL6 synthesis in immune cells compared with HCT116 cells. I then investigated whether miRNAs could be the factors released by IL6-treated CRC

cells involved in inducing IL6 production by THP1 cells.

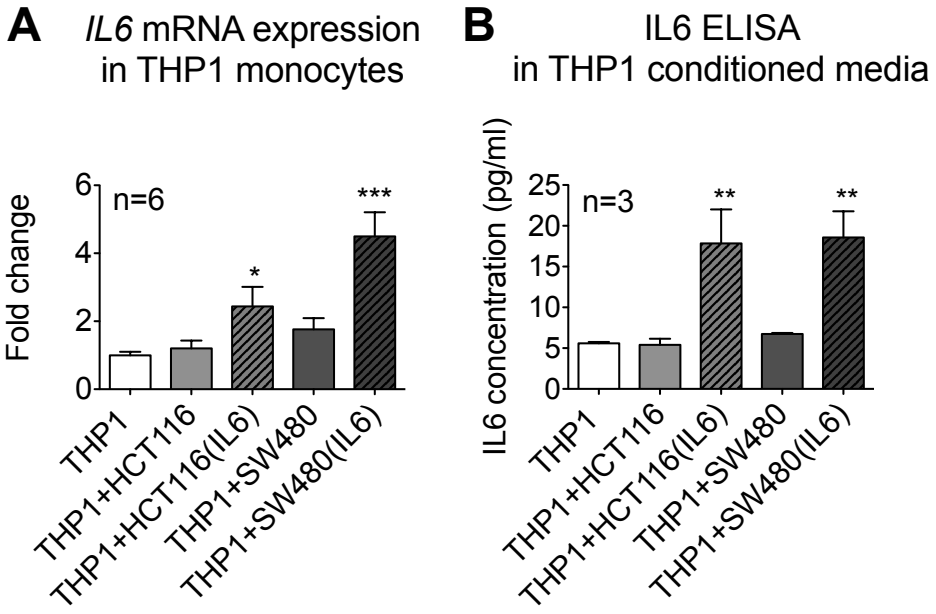

Figure 4.4 Effect of CRC cells on IL6 synthesis by THP1 monocyte-like cells. HCT116 and SW480 cells were pre-treated with or without 1000pg/ml IL6 for 24 hours prior to co-culturing with THP1 cells for a further 24 hours. (A) *IL6* expression in THP1 cells was measured by RT-qPCR (n=6). Data were normalised to expression of *GAPDH* and are shown relative to negative control (THP1 alone). (B) IL6 secretion by THP1 cells was measured in the conditioned medium using an ELISA (n=3). Data are expressed as fold change compared to negative control (THP1 alone). Significance was calculated using one-way ANOVA with a Dunnett post-test (GraphPad Prism 5, *p<0.05, **p<0.01, ***p<0.001). Error bars represent the SEM for independent cultures.

4.3.3 Are IL6-induced miRNAs involved in CRC-immune cell communication?

In the previous Chapter (Chapter 3, section 3.3.4), I determined that IL6 treatment of HCT116 and SW480 cells promoted expression of miR21 and miR29b. These two miRNAs have the potential to bind TLR8 to activate the immune cells and induce the NFκB pathway promoting IL6 expression. To investigate whether miR21 and miR29b are the factors secreted by IL6-treated CRC cells that promote THP1-mediated IL6 production, I first investigated

whether these miRNAs were secreted by the CRC cells (Figure 4.5). While miR21 and miR29b are secreted by CRC cells alone, their secretion is significantly increased when the cells are treated with IL6 and this secretion is greater in SW480 cells, which correlates with their increased ability to stimulate *IL6* expression in THP1 cells.

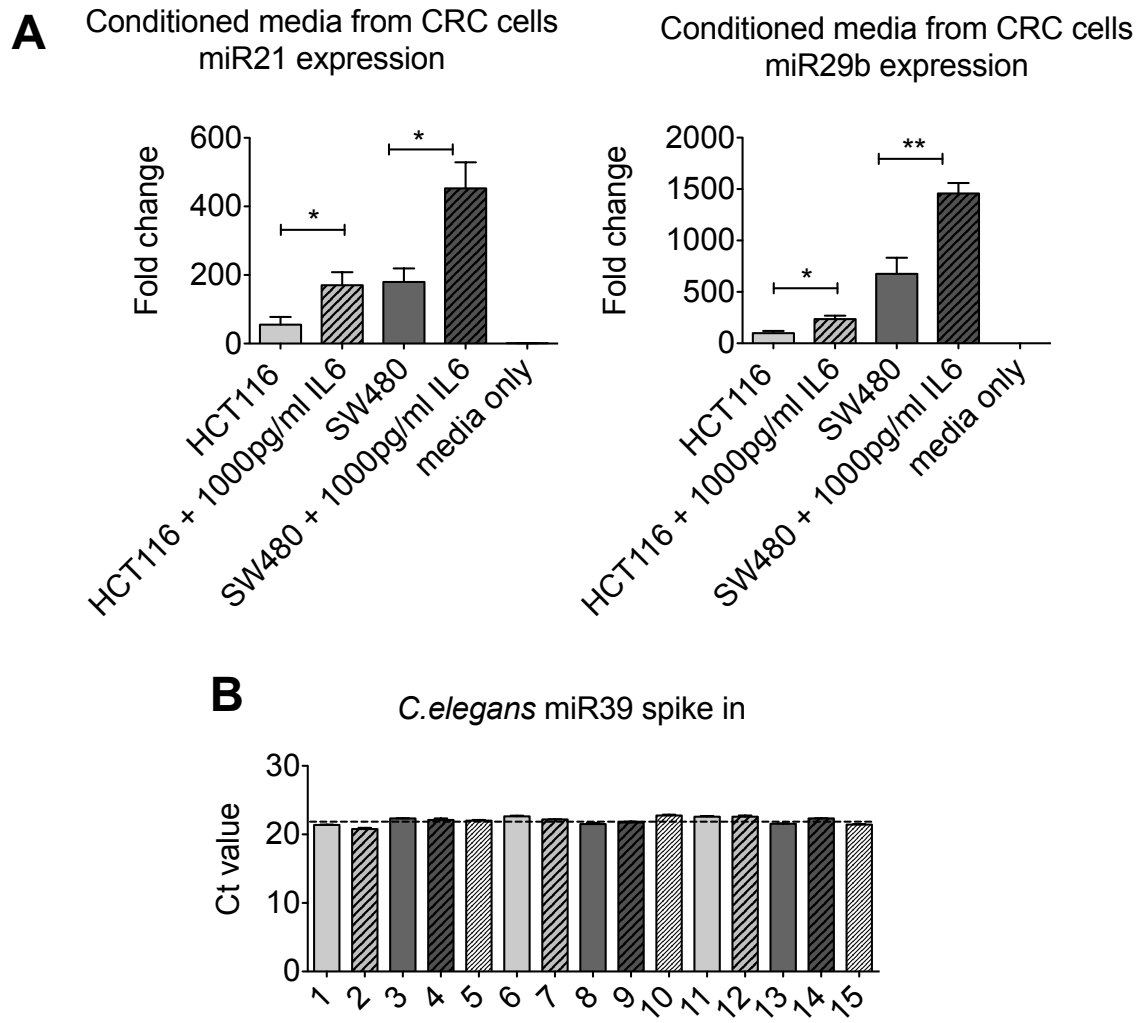

Figure 4.5 MiR21 and miR29b are secreted by IL6-treated CRC cells. HCT116 and SW480 cells were treated with or without 1000pg/ml IL6 and conditioned media from triplicate cultures was collected after 24 hours. (A) MiR21 and miR29b expression in the conditioned medium were measured by RT-qPCR. Data were normalised to expression of *C.elegans* miR39 spike-in (B). Data are expressed as fold change compared to negative control (unconditioned medium only). Significance was assessed using Student's *t*-test (GraphPad Prism 5, $*p<0.05$, $**p<0.01$). Error bars represent the SEM for independent cultures (n=3).

To confirm whether secreted miR21 and miR29b are able to induce *IL6* expression in receiving immune cells, miRNA mimics were transfected into THP1 cells and *IL6* mRNA expression was measured (Figure 4.6). Both miR21 and miR29b mimics induced more than a 2 fold induction of *IL6* expression in THP1 cells compared to the control mimic, suggesting that miR21 and miR29b secreted by IL6-treated CRC cells are able to further promote IL6 production in surrounding immune cells, thus generating a feedback loop.

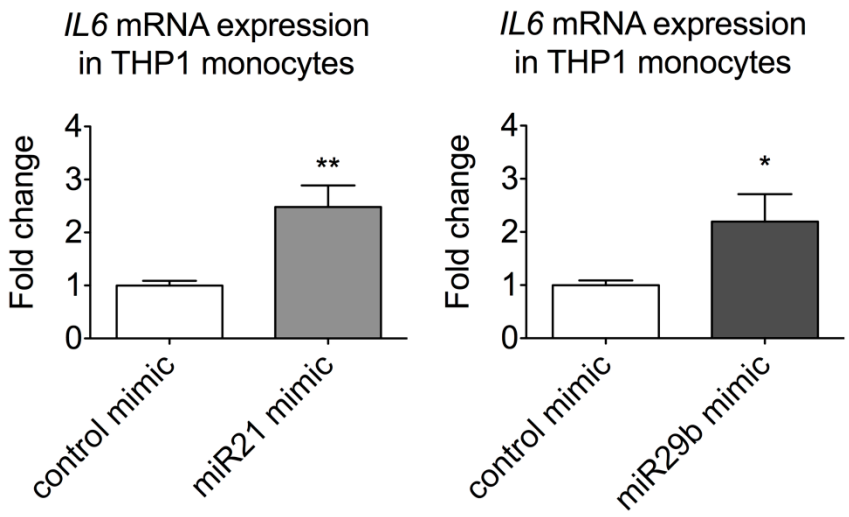

Figure 4.6 Effect of miR29b and miR21 on *IL6* expression in THP1 cells. MiR21 and miR29b mimics were transfected into THP1 cells. *IL6* mRNA expression was normalised to expression of *GAPDH*. Data are shown relative to mimic control. Significant differences were calculated using a Student's *t*-test (GraphPad Prism 5). Error bars represent the SEM for independent cultures (n=3).

Activated immune cells induce the NFκB pathway, which is known to promote miR21 expression (Shin *et al.* 2011). As miR21 is a circulating miRNA and known to be oncogenic, I sought to determine whether activated immune cells could also secrete miR21. To investigate this, conditioned medium from

stimulated THP1 cells was collected and measured for presence of miR21. Indeed, the latter was significantly increased in the conditioned medium of immune cells and was further induced when cells were activated by LPS (Figure 4.7). Therefore, miR21 secretion into the TME by immune cells could be involved in promoting cancer progression.

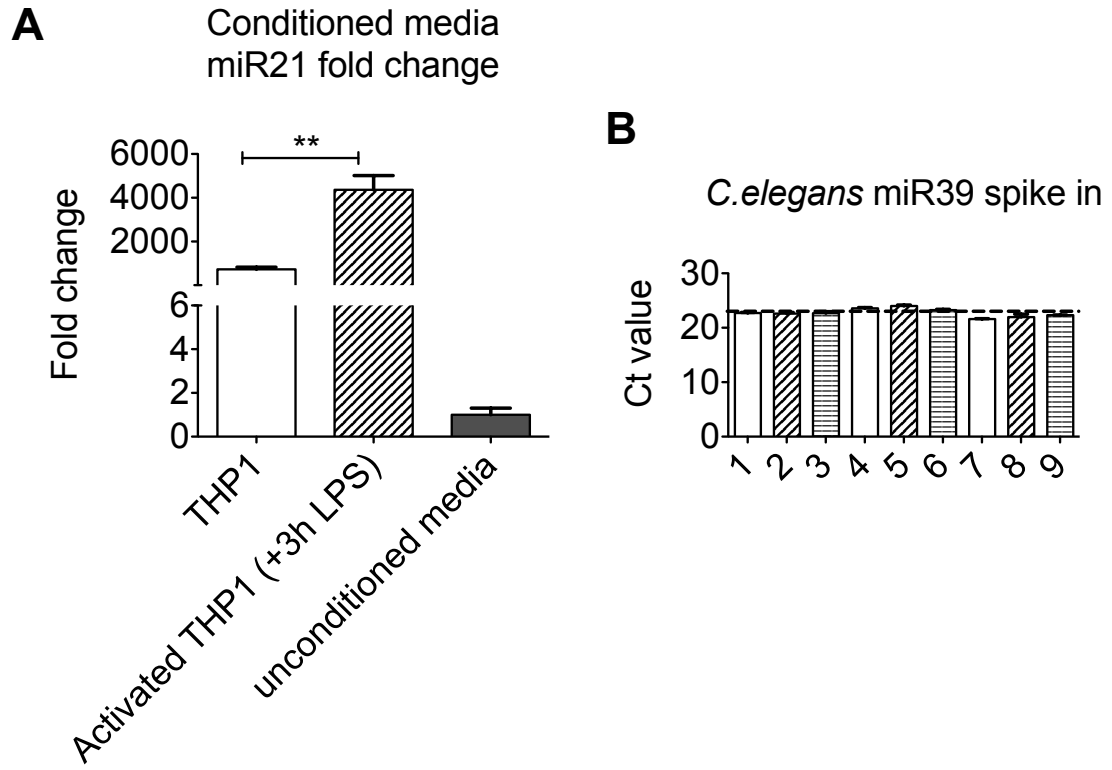

Figure 4.7 MiR21 is secreted by activated immune cells. THP1 cells were activated with 1µg/ml LPS for 3 hours and conditioned media from triplicate cultures was collected. (A) MiR21 expression in the conditioned medium was measured by RT-qPCR. Data were normalised to expression of *C.elegans* miR39 spike-in (B). Data are expressed as fold change compared to negative control (unconditioned medium only). Significance was calculated using Student's *t*-test (GraphPad Prism 5, $^{**}p<0.01$). Error bars represent the SEM for independent cultures (n=3).

4.4 Discussion

The TME is a dynamic and complex environment that is closely regulated by the tumour cells through extracellular signals. The tumour is thought to regulate its TME in order to promote its survival and progression. The tumour and its TME are therefore constantly interacting and miRNAs have emerged as new potential mediators of this crosstalk. Here, I demonstrate that IL6-treated CRC cells induced THP1-mediated IL6 production when co-cultured. My data suggest that miR21 and miR29b are secreted by CRC cells in response to IL6 and can promote surrounding immune cells to secrete IL6. Activated immune cells were also able to secrete oncogenic miR21 into the TME, which could signal back to the CRC cells and further promote tumour progression (Figure 4.8).

CM containing IL6 from LPS-treated THP1 cells promoted CRC cell invasion. Interestingly, IL6 alone was found to promote cell invasion to a similar level, suggesting that the effect of the CM is likely to be mediated by IL6, and this idea could be further confirmed by adding an IL6 antibody to the CM to block any IL6-mediated effect. Furthermore, when treated with LPS, undifferentiated monocyte-like THP1 cells are polarised towards a pro-inflammatory M1-like phenotype characterised by secretion of pro-inflammatory cytokines such as IL6. Previous studies have shown that pro-inflammatory tumour-infiltrating M1 macrophages have a tumour-suppressive effect due to their ability to elicit an anti-tumour immune response, and are thus associated with a better prognosis in CRC cases (Edin *et al.* 2012; Engström *et al.* 2014). On the other hand, IL6 is known to promote tumour growth and invasion (Schneider *et al.* 2000; Hsu & Chung 2006; Becker *et al.* 2005), and high levels of IL6 correlate with poor disease outcome (Chung & Chang 2003). The results from the current study

suggest that pro-inflammatory M1-like immune cells in the TME are able to promote cancer cell invasiveness, most likely through IL6 secretion. However, it is recognised that the current *in vitro* co-culture model only contains two cell types and thus fails to accurately replicate the complex TME of tumours *in vivo*. Therefore, in this simplistic model, the ability for activated M1-like THP1 cells to communicate with other immune cells (such as cytotoxic T cells and Th1 cells) and mount an anti-tumour response is eliminated, possibly explaining why they are observed to be tumour-promoting rather than tumour-suppressing here.

Unlike the previous studies presented in Chapters 2 and 3, 3D culture models were not used here. This is due to the fact that cells grown as 3D spheroids did not have significantly increased expression of miR21 and miR29b following IL6 treatment (discussed in Chapter 3, section 3.4). While the 3D cultured cells are reported to provide a more accurate representation of tumours *in vivo*, they would still fail at bridging the gap between 2D culture and *in vivo* here. As with 2D cell culture, only a limited number of different cell types can be used, whereas the TME *in vivo* is composed of signals from a multitude of cell types (detailed in Chapter 1, section 1.3) including endothelial cells, fibroblasts and various immune cells (Th1, Th2, cytotoxic T cells, NK cells, M1 and M2 macrophages). Therefore, studies looking at intercellular communication within the TME are best undertaken with *in vivo* models.

The current study used THP1 cells to mimic monocytes, however THP1 cells are derived from a patient with acute monocytic leukaemia and thus their behaviour does not always accurately mimic that of normal peripheral blood monocytes. Had time permitted, use of normal peripheral blood monocytes isolated from

healthy donors would have been informative. Studies have shown that while using the THP1 cell line provides valuable information on molecular mechanisms of monocytes and macrophages in various conditions, further validating findings using primary cells and *in vivo* models is important (Qin 2012; Schildberger *et al.* 2013).

Previous reports have shown that functional miRNAs can be secreted by cells and exert their effect in receiving cells. Valadi *et al.* were the first to demonstrate that exosomes from mouse and human cells contained not only protein but also mRNA and miRNA molecules, some of which were expressed at higher levels in the vesicles compared to their cell of origin, suggesting certain miRNAs are loaded specifically into vesicles while others are kept inside the cell (Valadi *et al.* 2007). These miRNA-containing exosomes are able to integrate into surrounding cells and release functional miRNAs (Montecalvo *et al.* 2012). More interestingly, Fabbri *et al.* reported that miR21 and miR29a were able to bind TLR8 to activate the NFκB pathway in recipient immune cells resulting in cytokine production (Fabbri *et al.* 2012). This binding event was dependent on the presence of a GU motif in the nucleotide region 18-21 of the miRNAs (Fabbri *et al.* 2012). MiR29b is closely related to miR29a and contains a GU motif in region 19-23 (GUGUU), thus it has the potential to interact with TLR8 in the tumour-surrounding immune cells. These previous reports thus support my finding that miR21 and miR29b mimics could induce *IL6* expression in recipient immune cells. However, the current study did not establish the exact mechanism by which miR21 and miR29b are able to do this. Although the study by Fabbri *et al.* suggests a mechanism by which the miRNAs activate NFκB signalling by binding to TLR8 (Fabbri *et al.* 2012), NFκB or

TLR8 knockdown studies in THP1 cells could be used in order to confirm whether the miRNAs signal through this pathway to mediate their effects. In addition, Fabbri *et al.* demonstrated in their model that the miRNAs were contained within exosomes (Fabbri *et al.* 2012), but here, the method of transfer of the miRNAs was not characterised. MiRNAs can be secreted by cells in various forms: they can be packaged in microvesicles and apoptotic bodies but can also be vesicle-free associated to AGO or HDL proteins (Turchinovich *et al.* 2013), thus an interesting further study would be to determine which of these methods the CRC cells use to secrete miR21 and miR29b using techniques such as ultra-centrifugation.

Furthermore, immune cells secreted miR21 upon activation. Indeed, LPS-mediated immune cell activation results in induction of the NFκB pathway, which is known to promote expression of a variety of pro-inflammatory cytokines including IL6 but also miRNAs such as miR21 (Shin *et al.* 2011). MiR21 is a known oncogenic miRNA, upregulated in a variety of cancer types (Asangani *et al.* 2008) and thus its secretion by surrounding immune cells could be another mechanism (in addition to cytokine secretion) by which these cells are able to promote tumour progression. Moreover, secreted oncogenic miRNAs (by immune or tumour cells) could integrate into nearby normal cells and promote their cellular transformation. A better understanding of the mechanisms involved in cellular communication within the TME is essential, and could potentially lead to the development of new therapeutic strategies.

4.5 Summary

The current study demonstrates that LPS-activated THP1 cells secrete IL6 and miR21, which can induce adjacent tumour cell invasion thus promoting tumour progression. The findings also suggest that IL6-treated tumour cells can interact with immune cells through secretion of miR21 and miR29b, resulting in further secretion of IL6 by the immune cells (Figure 4.8). Although *in vitro* co-culture models are unable to accurately replicate the complexities of the TME *in vivo*, the findings from the current model do offer mechanistic support for a potential role for IL6 and miRNAs as mediators of cancer-immune cell crosstalk.

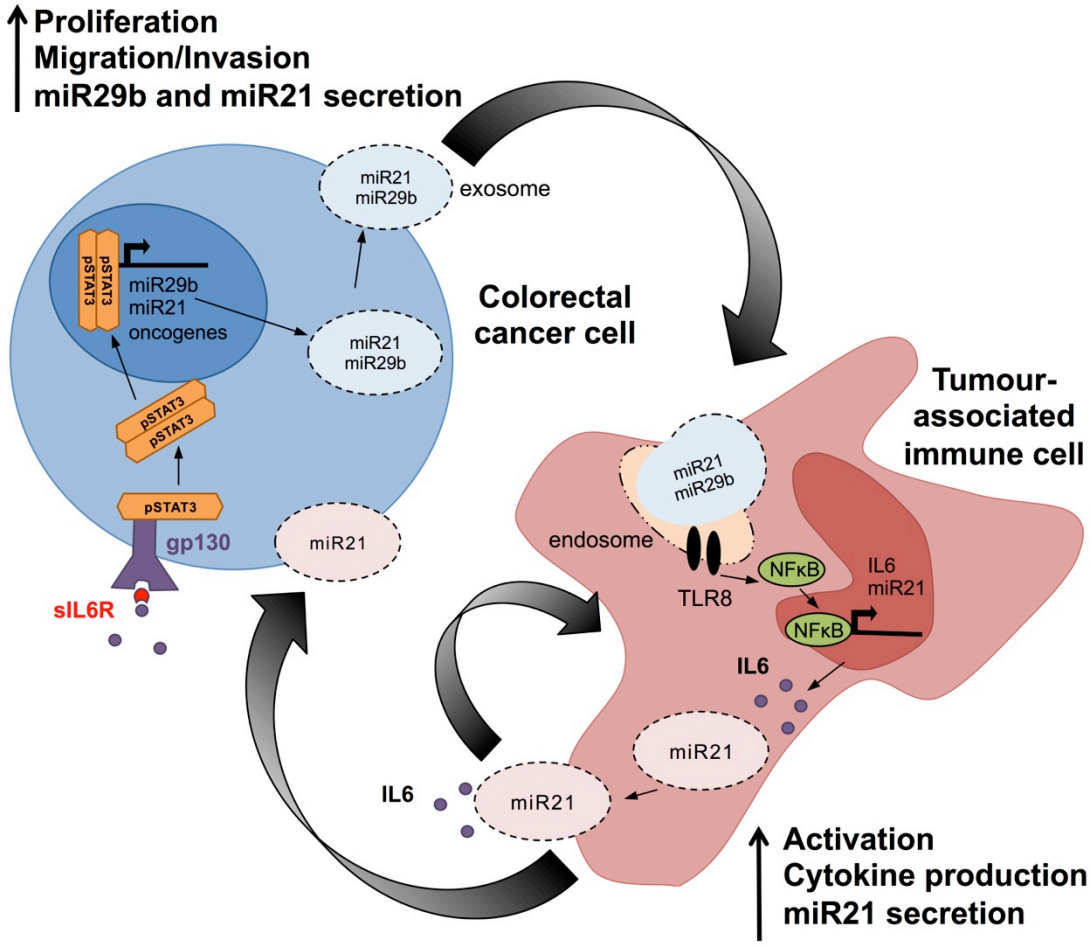

Figure 4.8 Proposed model for IL6-mediated CRC and tumour-associated immune cell crosstalk involving miR21 and miR29b. IL6 is secreted by tumour-associated immune cells. Upon binding to IL6R on the colorectal cancer cell surface, pSTAT3 is translocated to the nucleus to induce expression of a number of oncogenes and miRNAs including miR21 and miR29b, which are then secreted into the TME *via* exosomes and are taken up by the tumour-associated immune cells. MiR21 and miR29b then bind to TLR8 within the immune cells sustaining their activation and inducing further secretion of IL6 and miR21 into the microenvironment to promote cancer cell progression.

- Chapter 5 -

Discussion

5.1 IL6 promotes environment-associated CRC: mechanisms and preventive/therapeutic strategies.

In the current project, IL6 was shown to promote BaP and PhIP activation in CRC cells through induction of *CYP1B1* expression. The latter was induced by IL6-mediated downregulation of miR27b expression through a mechanism involving DNA methylation. IL6 is thus able to induce epigenetic changes that promote dietary carcinogen-induced DNA damage. IL6 also induced expression of *CYP2E1*, another CYP450 enzyme known to metabolise drugs, however this mechanism did not appear to involve miRNAs but instead was mediated by a direct transcriptional regulation by STAT3 (Chapter 2).

These CYP450 enzymes can also activate carcinogens from other environmental factors such as pollution or smoking (Crofts *et al.* 1997; Gautier *et al.* 1996; Shimada *et al.* 1997; Bolt *et al.* 2003; Lu & Cederbaum 2008). Smokers have been shown to have high plasma levels of IL6 (Ridker *et al.* 2000), thus these novel IL6-mediated CYP450 regulatory mechanisms could also be important in environment-associated CRC in general. As mentioned in the introduction (Chapter 1), CRC is widely regarded as a lifestyle disease and thus preventing IL6-mediated expression of CYP450s could potentially be an effective disease prevention strategy.

In addition to foods containing carcinogens, consumption of high levels of alcohol has been associated with increased CRC risk (Pollack *et al.* 1984; Seitz *et al.* 1984; Kune *et al.* 1987) and increased levels of IL6 occur with alcohol consumption (Hong *et al.* 2002; Martinez *et al.* 1992), thus alcohol-promotion of CRC could involve IL6. Interestingly, ethanol can increase CYP2E1 expression

although the specific molecular mechanisms are not yet clear (Lieber 1997). This novel CYP2E1 regulatory pathway involving IL6 and STAT3 could provide a potential mechanism.

CYP450 enzymes are also involved in metabolising a wide array of drugs, thus identifying mechanisms of their regulation in tumours could have significant implications in cancer therapies. High levels of IL6 at the tumour site has been associated with multiple drug resistance in a variety of cancer types (Ara *et al.* 2013; Yan *et al.* 2014). IL6-mediated induction of local CYP450 expression could be involved in this effect as these enzymes could inactivate chemotherapeutic drugs, thus administrating selected drugs that are not inactivated by these enzymes or combining drugs with an anti-IL6 adjuvant therapy could potentially attenuate drug resistance. Another strategy would be using the increased CYP450 expression at the tumour site to improve drug specificity; indeed inflammation-associated cancers could also benefit from drugs that are selectively activated by either CYP1B1 or CYP2E1, and thus are preferentially targeting the cancer cells. In this case, drugs activated by CYP1B1 would be a better option as this enzyme has been shown to have tumour-specific expression (Murray *et al.* 1997; Rochat *et al.* 2001) whereas CYP2E1 is also expressed at relatively high levels in hepatic tissue (Tan *et al.* 2001).

While I have only investigated these IL6-mediated effects in CRC cells, this pathway could also be involved in other environment- and lifestyle-induced cancers such as lung, breast or prostate, and therefore similar prevention or therapeutic strategies could apply in these cases.

Furthermore, regular intake of NSAIDs has been associated with lower cancer risk including CRC, breast and lung, however the specific underlying mechanisms remain unclear (Smalley *et al.* 1999; Olsen *et al.* 2008; McCormack *et al.* 2011; Rothwell *et al.* 2011; Rothwell, Price, *et al.* 2012; Rothwell, Wilson, *et al.* 2012; Algra & Rothwell 2012). Prevention of IL6-mediated induction of *CYP1B1* and *CYP2E1* expression may be partially responsible, for this effect. In addition, preventing other IL6-mediated cancer promoting pathways such as IL6 promotion of cancer cell metastatic behaviour, activation of STAT3 and upregulation of oncogenic miRNAs such as miR21, could also be responsible for NSAIDs chemoprotective properties (Figure 5.1).

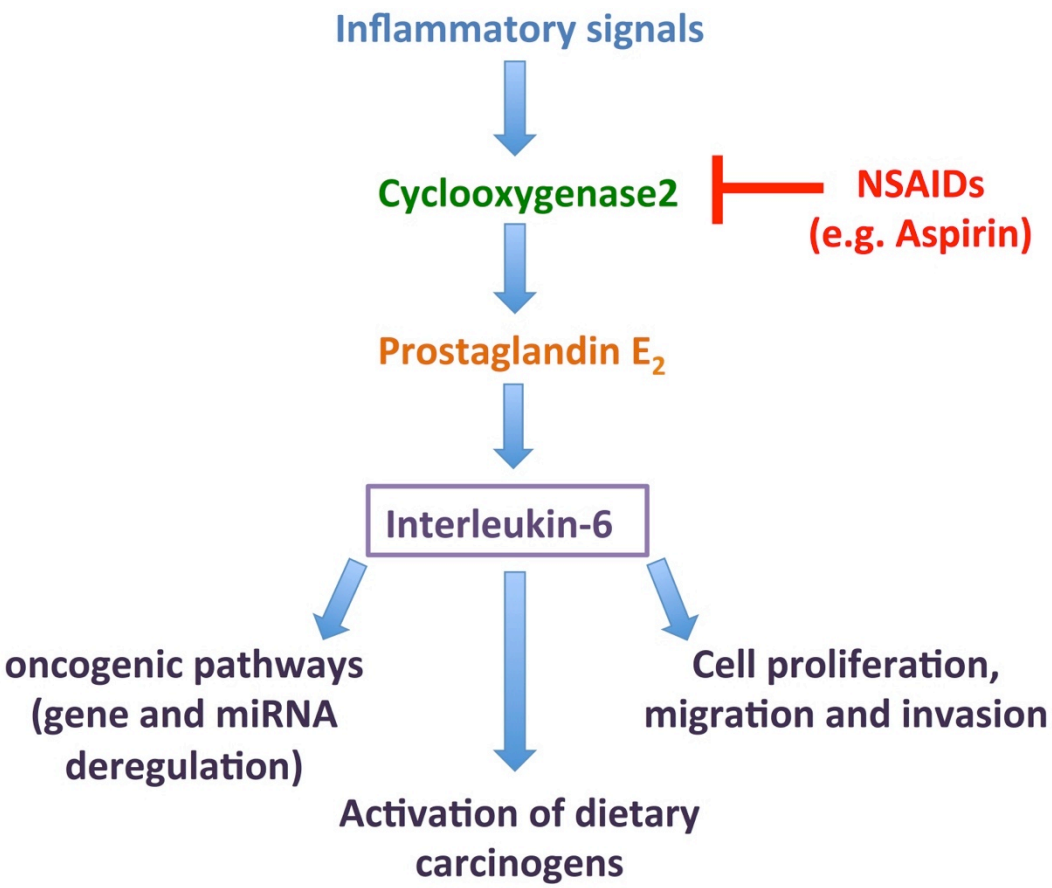

Figure 5.1 NSAIDs lower CRC risk by inhibiting IL6 production. NSAIDs inhibit cyclooxygenase-2 that catalyses prostaglandin E_2 synthesis, which in turn regulates IL6 synthesis in immune cells. Adapted from Williams & Shacter 1997.

5.2 The role of IL6 in CRC progression: more than just STAT3 activation?

It has been suggested that IL6 could promote cancer progression primarily *via* STAT3 activation. Indeed, activation of the JAK/STAT3 pathway occurs in most cancer cells and results in transcription of a number of oncogenes that promote cell survival, proliferation, angiogenesis and metastasis (Teng *et al.* 2014; Du *et al.* 2012; Becker *et al.* 2005; Rokavec *et al.* 2012; Wei *et al.* 2003; Rokavec *et al.* 2014); therefore STAT3 has emerged as a promising therapeutic target for cancer.

In the current project, IL6 was shown to promote cell metastatic behaviour through increased expression of oncogenes and miRNAs (Chapter 3). Interestingly, inhibition of STAT3 signalling was able to prevent these effects, suggesting that STAT3 plays a central role in IL6-mediated CRC promotion. As mentioned previously, STAT3 was also found to directly regulate *CYP2E1* expression, which could potentially be involved in dietary carcinogen activation and chemotherapeutic drug resistance. The essential role of STAT3 in CRC progression has also been demonstrated *in vivo* in mouse models for colitis-associated CRC, where lack of STAT3 resulted in significant reduction of tumour size and tumour burden (Bollrath *et al.* 2009; Grivennikov *et al.* 2009). However, lack of STAT3 did not completely prevent tumour formation. While STAT3 appears to be a crucial part of IL6-mediated CRC promotion, it is not the only mechanism by which IL6 exerts its effects. Indeed, data from the current project demonstrates that IL6 can promote dietary carcinogen-induced DNA damage through induction of CYP1B1 expression in a mechanism independent of STAT3 activation, as inhibition of STAT3 signalling did not prevent this effect

(Chapter 2). Genome-wide methylation is a known effect of IL6 (Yan *et al.* 2011; Wehbe *et al.* 2006) and is thought to be mediated by increased expression of DNMT1 through AKT activation (Hodge *et al.* 2007) resulting in altered expression of a number of tumour-suppressors and oncogenes. IL6 signalling is also known to activate a number of other tumour-promoting pathways such as PI3K/AKT and MAPK/ERK, and these pathways have been shown to be essential to IL6-mediated cancer progression (Zhang *et al.* 2013; Lo *et al.* 2011; Zhang *et al.* 2003; Wegiel *et al.* 2008). Therefore, while STAT3 appears to be a promising drug target for cancer therapy, the question arises: is inhibiting STAT3 sufficient or would anti-IL6 therapy be more effective? The findings from the current project and other studies (Zhang *et al.* 2013; Lo *et al.* 2011; Zhang *et al.* 2003; Wegiel *et al.* 2008) suggest the latter.

Numerous anti-IL6 therapies have emerged (Figure 5.2). Amongst these, a monoclonal anti-IL6 antibody, siltuximab, is currently in phase I/II clinical trials although the initial studies have provided mixed results (Karkera *et al.* 2011; Guo *et al.* 2010; Rossi *et al.* 2010) possibly due to the fact that antibody-associated IL6 is not cleared from the circulation (Waldner *et al.* 2012; Jones *et al.* 2011). Another strategy is to target IL6R; an anti-IL6R antibody (tocilizumab) is and has shown promising effects in clinical trials for treating chronic inflammatory conditions such as arthritis (Sato *et al.* 1993; Tanaka *et al.* 2012). However, these therapies also inhibit the physiological effects of IL6 and could have significant adverse effects. A more targeted strategy is to selectively inhibit IL6 *trans*-signalling, which is the pathway primarily involved in cancer progression (Scheller *et al.* 2006). To this effect, an inhibitor that can

bind to the IL6/sIL6R complex has been designed (spg130Fc) and is currently in preclinical trials (Waetzig & Rose-John 2012).

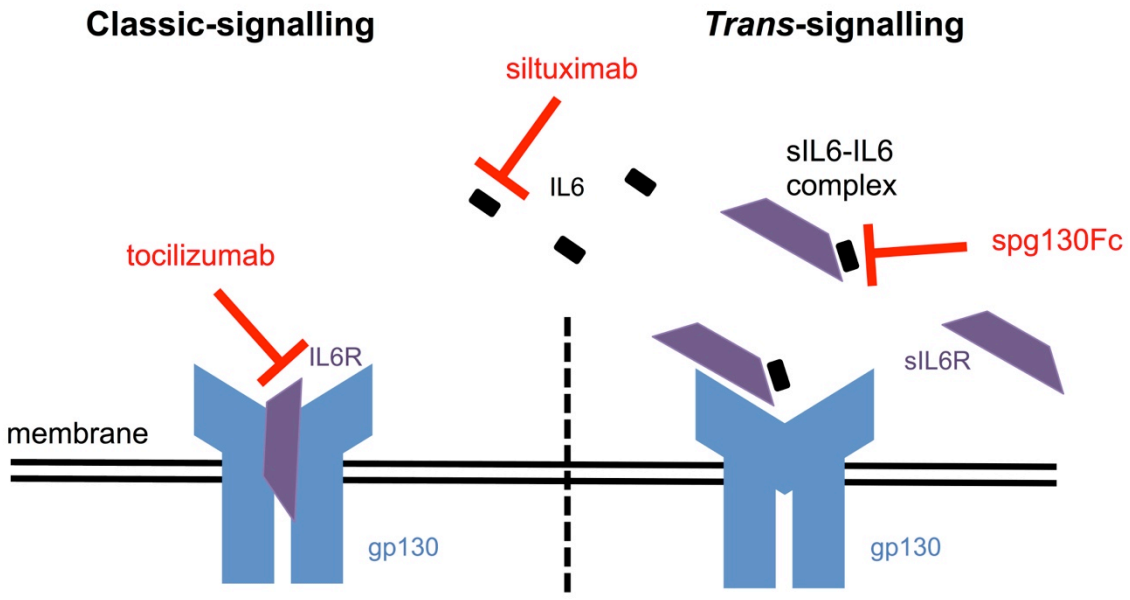

Figure 5.2 Anti-IL6 therapies in clinical trials.

In addition, in the current project, I have demonstrated the diverse roles of miRNAs in IL6-mediated CRC progression. A novel therapeutic strategy would be to use miRNAs to inhibit IL6 effects. MiRNA mimics could be used to target JAK or gp130 expression. The production of sIL6R by metalloproteinases of the ADAM family (ADAM10 and ADAM17; Briso *et al.* 2008) could potentially also be targeted by miRNA therapy to specifically inhibit IL6 *trans*-signalling. A miRNA mimic for miR34 has recently gone into phase I clinical trial for liver cancer (Agostini & Knight 2014), suggesting miRNA mimic therapy as a promising strategy for future cancer therapies.

5.3 Role of miRNAs in IL6-mediated CRC progression

This project investigated the mechanisms by which IL6 can promote CRC progression with a focus on miRNA involvement. Given the current findings, it appears that miRNAs play an essential role in the various pathways of IL6-mediated CRC promotion.

Indeed, miR27b downregulation is responsible for *CYP1B1* expression changes resulting in increased activation of dietary carcinogens and DNA damage. While miR27b expression changes were investigated due to its ability to regulate *CYP1B1* expression (Tsuchiya *et al.* 2006), this miRNA appears to have other tumour-suppressive functions in various cancer types including CRC by targeting oncogenes such as VEGFC, TCPT and PPARγ (Lee *et al.* 2012; Lo *et al.* 2012; Ye *et al.* 2013). Therefore, its downregulation by IL6 may have other tumour-promoting consequences in addition to increased dietary carcinogen-induced DNA damage.

Furthermore, this study demonstrated that IL6 induced expression and secretion of miR21 and miR29b in CRC cells. While the role of miR29b in cancer remains controversial (see discussion section in Chapter 3), it appears to be promoting tumour-like activity in the current study. Interestingly, miR29b has been shown to target the DNMT3 family of enzymes (Garzon *et al.* 2009); therefore regulation of miR29b by IL6 could be contributing to IL6-mediated global DNA methylation changes observed in previous studies (Hodge *et al.* 2005; Wehbe *et al.* 2006; Li *et al.* 2012). It also appears to play a pro-inflammatory role in the CRC TME by promoting IL6 secretion by adjacent immune cells. MiR21 on the other hand is a well-known oncogenic miRNA

known to downregulate a number of tumour-suppressor genes such as *PTEN* and *PDCD4* (Asangani *et al.* 2008; Peacock *et al.* 2014; Meng *et al.* 2007). MiR21 expression is increased in nearly all cancer types but also in numerous infections as it is regulated by inflammatory pathways STAT3 and NFκB (Shin *et al.* 2011; Yang *et al.* 2010; Ma *et al.* 2013). It was therefore not surprising that miR21 expression and secretion were increased in response to IL6 and may contribute to the IL6 cancer promoting effects. Interestingly, miR21 is also upregulated in response to a variety of risk factors for CRC such as chronic inflammation, age, obesity, Western-style diet and smoking (Melnik 2015), suggesting that miR21 may be involved in the mechanisms by which these factors promote CRC initiation and progression. In addition, as with miR29b, miR21 plays a pro-inflammatory role in the CRC TME where it is not only secreted by IL6-stimulated cancer cells to induce immune cell IL6 synthesis but also secreted by activated immune cells. Due to its oncogenic functions, miR21 has emerged as a promising drug target (Chan *et al.* 2014; Sicard *et al.* 2013) and could be particularly useful for inflammation-associated cancers including CRC. While therapeutic delivery of a miR21 inhibitor remains challenging (Sicard *et al.* 2013), the current findings along with previous research suggest that inhibiting IL6 signalling would result in miR21 downregulation along with inhibition of other cancer-promoting pathways.

5.4 Limitations of study and future work

The current project was performed predominantly using *in vitro* cell culture models. While these models are appropriate for mechanistic studies, they do not provide an accurate representation of tumours *in vivo* and often results observed *in vitro* do not translate *in vivo*. As an attempt to bridge the gap between *in vivo* and *in vitro*, 3D cell culture was used to verify observations in 2D cell culture (Pampaloni *et al.* 2007) and while this provides some validation, it does not prove *in vivo* relevance. A comparison of cellular behaviour between 2D and 3D cell culture systems can be found in appendix C. While many of responses observed in 2D culture were reproducible using 3D cultures, some IL6-mediated effects were absent in 3D culture such as miR21 and miR29b regulation. These discrepancies emphasise the need to explore these questions further using *in vivo* models. In addition, the use of *in vivo* models remains the most thorough approach to studying TME with all its complexities.

Using *in vitro* methods implies the use of cell lines. In the current study, immortalised epithelial cancer cell lines derived from CRC patients were used. Due to the immortalisation and culture processes, cell lines are inclined to drift from their original genotypic and phenotypic state particularly in the case of cancer cells as they are genetically unstable to begin with (Pan *et al.* 2009), thus cultured cells have many limitations. As most cell lines are established from cancer cells, investigating cancer-initiating events is difficult. While a few cell lines from non-malignant cells exist, these undergo similar immortalisation processes (including phenotypic and genotypic drifting) and can also be challenging to grow in large numbers. More importantly, cell lines that are cultured for long periods in laboratory settings are not only susceptible to

phenotypic and genomic alterations, but also to contamination by mycoplasma, bacteria and other cells. While cell lines are usually authenticated by cell banks (such as ATCC) prior to purchase, it is necessary to re-authenticate cell lines regularly. This can be done by routine cell morphology monitoring, short tandem repeat profiling and karyotyping. The cell lines used in this study were not subjected to this level of re-authentication, although they were assessed by examining their growth rate and morphology, as well as confirming their mycoplasma-free status.

Another limitation of the current study is the lack of 'omic' investigation. Indeed, due to research budgetary restrictions, only targeted qPCR for specific genes and miRNAs were performed in response to IL6. These targets were informed by available literature on IL6 and CRC, and were a starting point to investigate the research hypothesis. However, this approach does not provide a complete picture of the effect of IL6 on the whole genome and miRNAome, and important responses are likely to be missed.

Given these limitations, follow-up studies could include verifying the IL6-mediated pathways identified in this project *in vivo*, investigating the role of IL6 on cancer initiation using both primary cells and *in vivo* models, and determining the IL6 effect on whole genome and miRNAome expression.

Having determined miRNA changes in response to IL6, future work could expand on determining the role of these miRNAs in CRC and whether manipulating these could have an effect on the cancer cells to identify potential miRNA therapeutic targets. This approach was successfully employed in

investigating the expression of CYP450s in colorectal tissue (see appendix D; Patel *et al.* 2014)

As the current project has provided evidence that IL6 may be involved in promoting environment-associated CRC, these pathways could be important in other environment-associated cancer types such as breast, prostate and lung. Therefore, an extension of this project would be to investigate these pathways in other cancer types and determine whether the observed effects are specific to CRC or apply to other solid tumour types.

While the current project focused on IL6 due to its central role in inflammation and CRC, other members of the IL6 cytokine family may have similar effects to those determined in this project. Indeed, oncostatin M, a member of the IL6 family of cytokines, is overexpressed in cancer tissue and is more potent at activating STAT3 than IL6 (Lapeire *et al.* 2014). Also, the TME is a very dynamic and multivariate milieu, and IL6 is only one of the molecules present in the TME. As described previously (Chapter 1, section 1.3 and Figure 1.5), there are numerous signals within the TME including other cytokines, chemokines and growth factors, and investigating these other signals is crucial to understand which of these plays a key role in promoting cancer progression and develop effective TME-targeting therapies.

Finally, this project has determined that miRNAs miR21 and miR29b (in response to IL6) potentially play an important role in the TME by mediating intercellular crosstalk between cancer and immune cells. However, it needs to be recognised that the immune cells (THP1) used in this study were derived from a leukaemia patient and thus differ from monocytes present in the TME,

therefore using primary non-malignant cells would provide further validation of the results found here. Further studies could also include determining the role of other miRNAs in the TME in a variety of cancer types. Another interesting aspect would be to investigate whether these secreted miRNAs can promote transformation of surrounding non-malignant cells.

5.5 Conclusions

As described in the Introduction (Chapter 1), CRC development and progression appear to be linked to lifestyle (particularly diet) and inflammation, however the underlying mechanisms remain unclear. The main hypothesis of this project was that the presence of pro-inflammatory cytokine IL6 in the TME could promote CRC progression by inducing epigenetic and genetic changes, and that these changes occur partially through local activation of dietary carcinogens and miRNA deregulation. This project generated a number of findings (presented and discussed in Chapters 2-4) identifying three different mechanisms by which IL6 can promote CRC progression, and miRNAs were found to play an important role in each of these effects. First, IL6 promoted dietary carcinogens BaP- and PhIP-induced DNA damage by promoting their activation pathway. Second, IL6 induced CRC cell behavioural changes by stimulating cell proliferation, migration and invasion. Last, IL6 mediated cell-cell communication between immune and CRC cells *via* miRNAs to maintain pro-inflammatory signals that promote CRC cell invasion. My studies are based on *in vitro* experimentation using cell models, which cannot accurately recapitulate the complexity of tumours *in vivo*. Therefore, additional studies are necessary to not only establish *in vivo* relevance but also determine whether these findings could be further investigated for therapeutic development. In conclusion, the findings of this project offer experimental mechanistic support for the proposed hypothesis and although *in vivo* significance needs to be demonstrated, these results do provide further insight into the potential mechanisms by which IL6 can promote CRC progression.

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Appendices

Appendix A: QPCR reference genes and expression assays

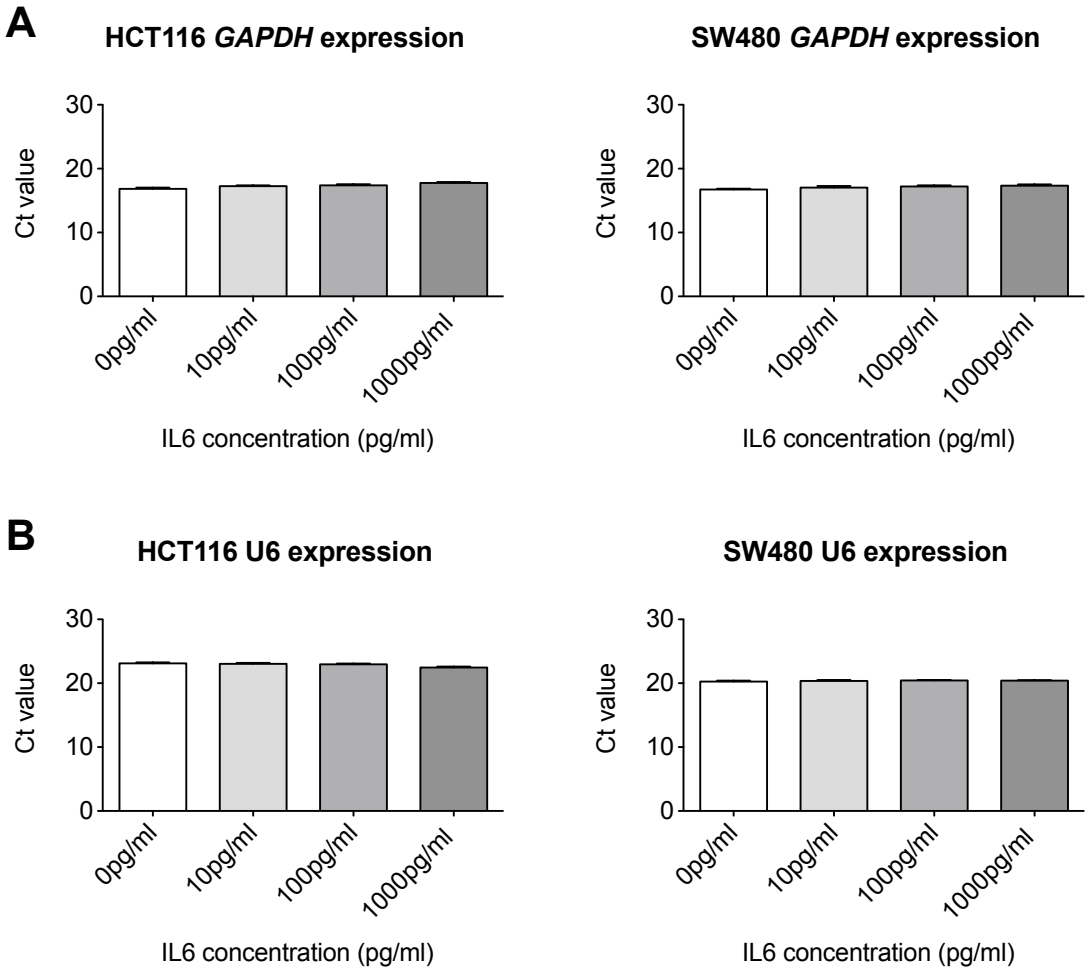

Figure S.1 GAPDH and U6 expression with IL6 treatment. GAPDH and U6 expression were measured by RT-qPCR in HCT116 and SW480 cells following 24 (SW480) and 48 hour (HCT116) IL6 treatment. Data are presented as mean Ct value. Error bars represent the SEM for independent cultures (n=3).

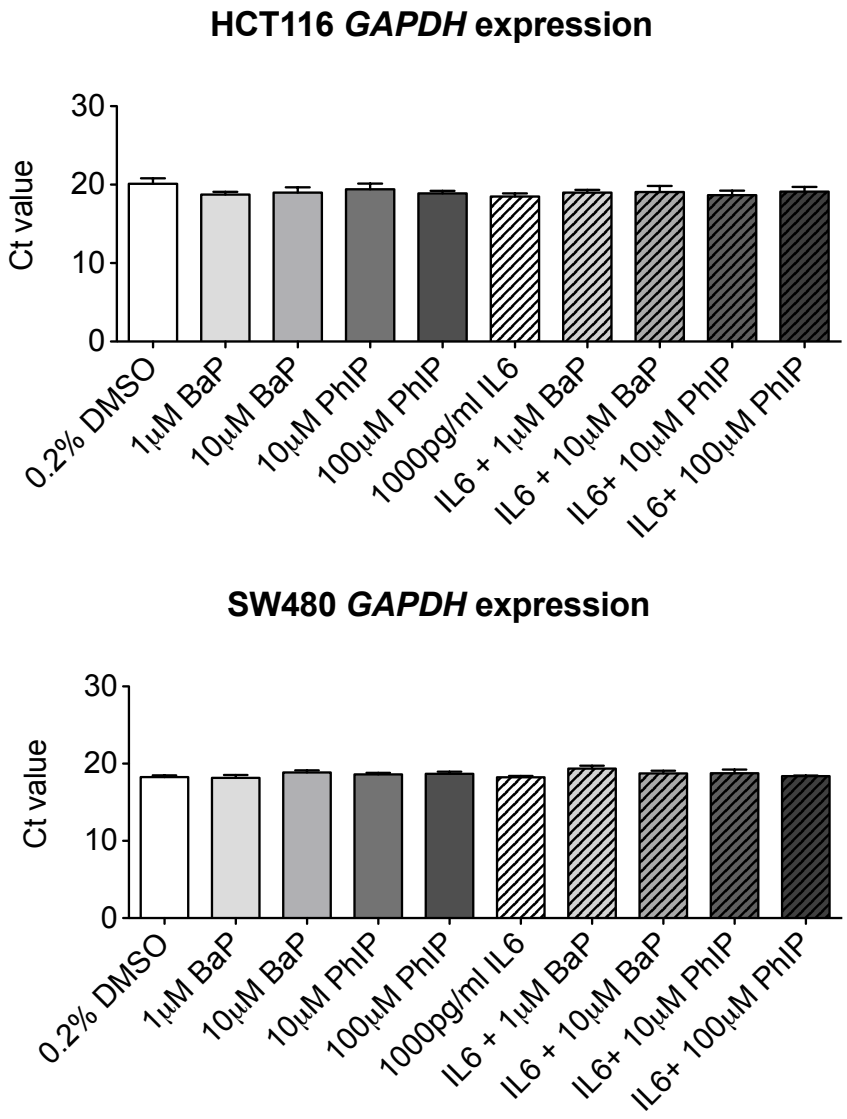

Figure S.2 *GAPDH* expression with BaP and PhIP treatment. *GAPDH* expression was measured by RT-qPCR in HCT116 and SW480 cells following BaP and PhIP treatment with or without IL6 pre-treatment. Data are presented as mean Ct value. Error bars represent the SEM for independent cultures (n=3).

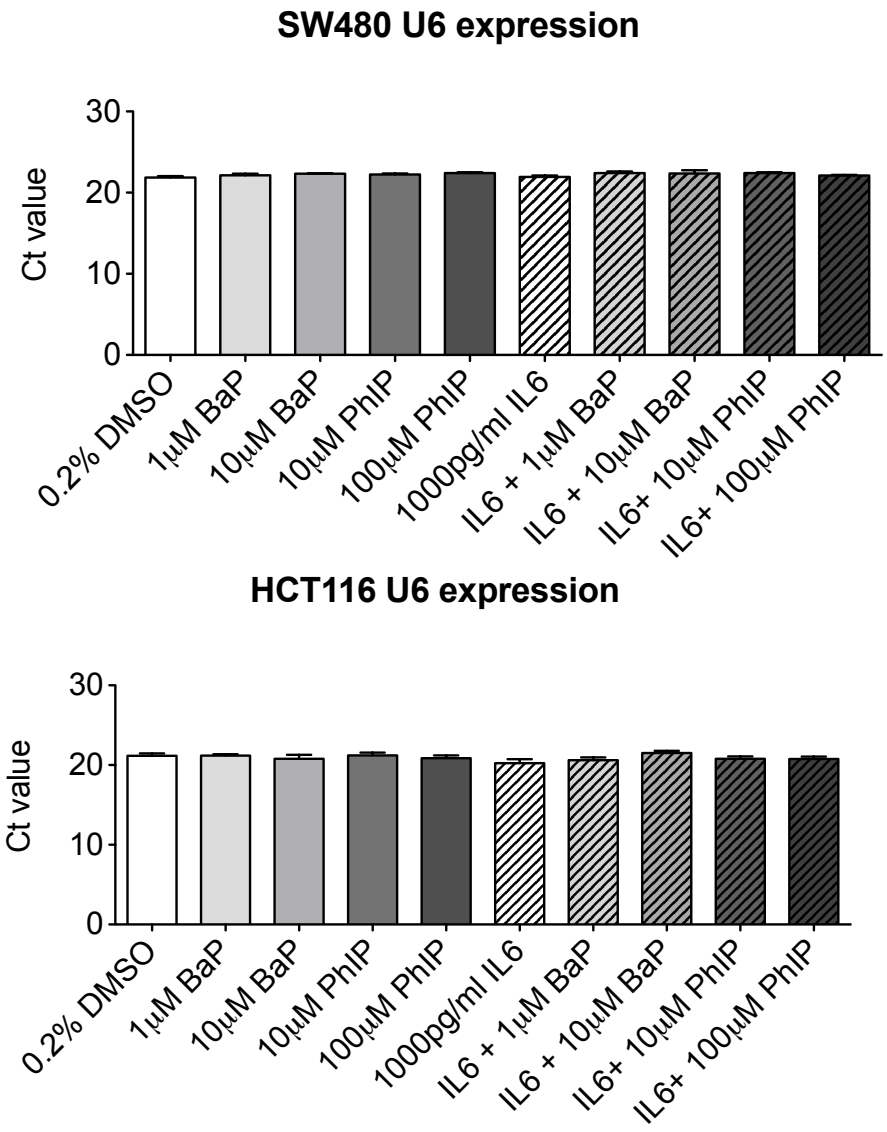

Figure S.3 U6 expression with BaP and PhIP treatment. U6 expression was measured by RT-qPCR in HCT116 and SW480 cells following BaP and PhIP treatment with or without IL6 pre-treatment. Data are presented as mean Ct value. Error bars represent the SEM for independent cultures (n=3).

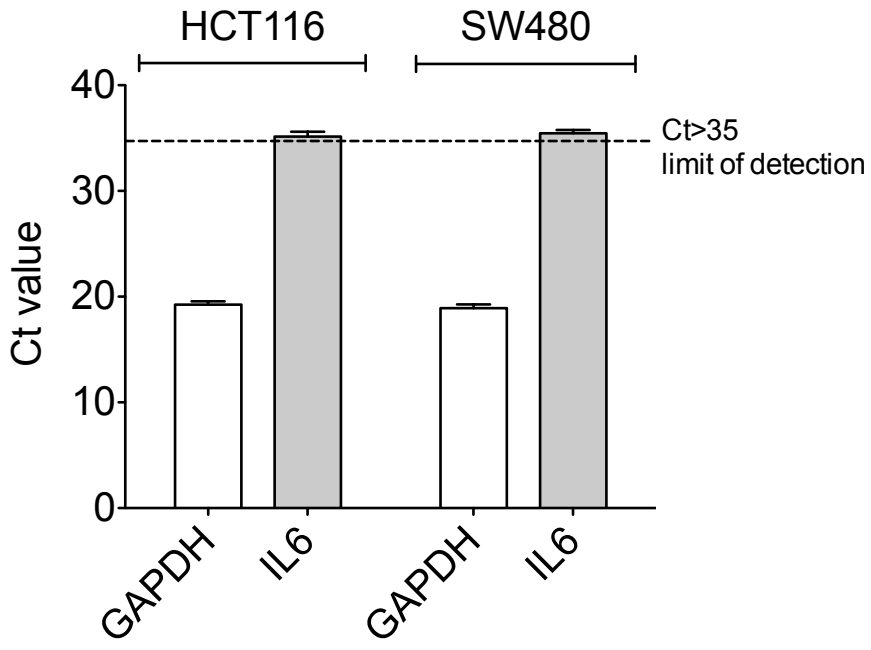

Figure S.4 *IL6* mRNA expression is not detected in HCT116 and SW480 cells. *IL6* and *GAPDH* expression were measured by RT-qPCR in HCT116 and SW480 cells. Reactions using Taqman assays with Ct>35 are excluded from further analyses as these values approach the sensitivity limit of the real-time PCR system and are thus considered unreliable. Data are presented as mean Ct value. Error bars represent the SEM for at least three independent cultures.

Table S1. Taqman real-time qPCR probes used in this study.

Gene/miRNA	Species	Taqman assay ID
CYP1A1	*H. sapiens*	Hs01054797_g1
CYP1B1	*H. sapiens*	Hs00164383_m1
CYP2E1	*H. sapiens*	Hs00559368_m1
p53	*H. sapiens*	Hs01034249_m1
E-cadherin	*H. sapiens*	Hs01023894_m1
VEGFA	*H. sapiens*	Hs00900054_m1
JAK2	*H. sapiens*	Hs00234567_m1
CTSD	*H. sapiens*	Hs00157205_m1
MMP2	*H. sapiens*	Hs00234422_m1
IL6	*H. sapiens*	Hs00174131_m1
GAPDH	*H. sapiens*	Hs99999905_m1
miR-27b-3p	*H. sapiens*	000409
miR-378-5p	*H. sapiens*	000567
miR-31-5p	*H. sapiens*	002279
let7a-5p	*H. sapiens*	000377

Gene/miRNA	Species	Taqman assay ID
miR-39	*C. elegans*	000200
miR-135b-5p	*H. sapiens*	002261
miR-96-5p	*H. sapiens*	000186
miR-124a-3p	*H. sapiens*	001182
miR-375	*H. sapiens*	000564
miR-21-5p	*H. sapiens*	000397
miR-29b-3p	*H. sapiens*	000413
U6	*H. sapiens*	001973

Appendix B: Expression of pro-inflammatory cytokines in CRC tissue

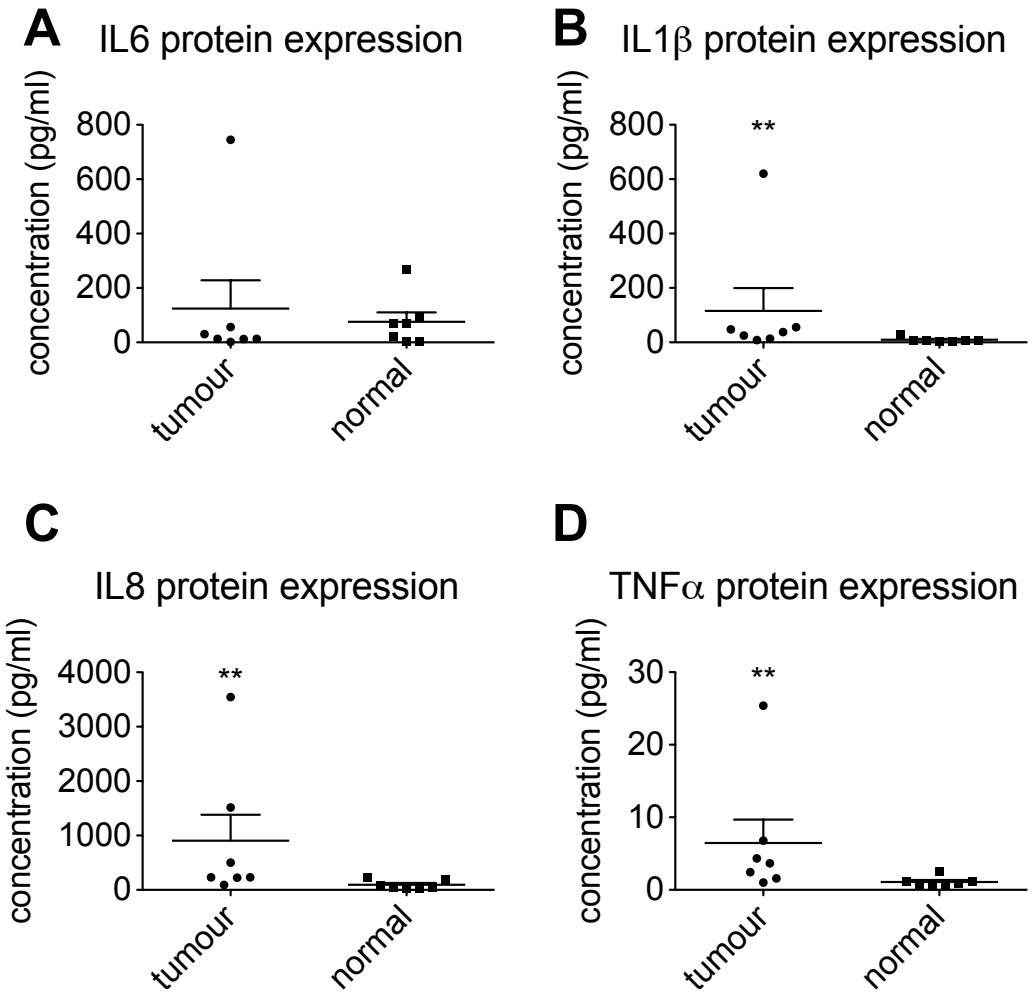

Figure S.5 Pro-inflammatory cytokine expression in CRC tissue. Cytokine concentration was measured in protein extracted from tissue samples and measured using the MSD platform technology. Data are presented as a mean cytokine concentration (pg/ml). Significant differences between tumour and normal tissue were calculated using a Mann-Whitney test (GraphPad Prism 5, n=7).

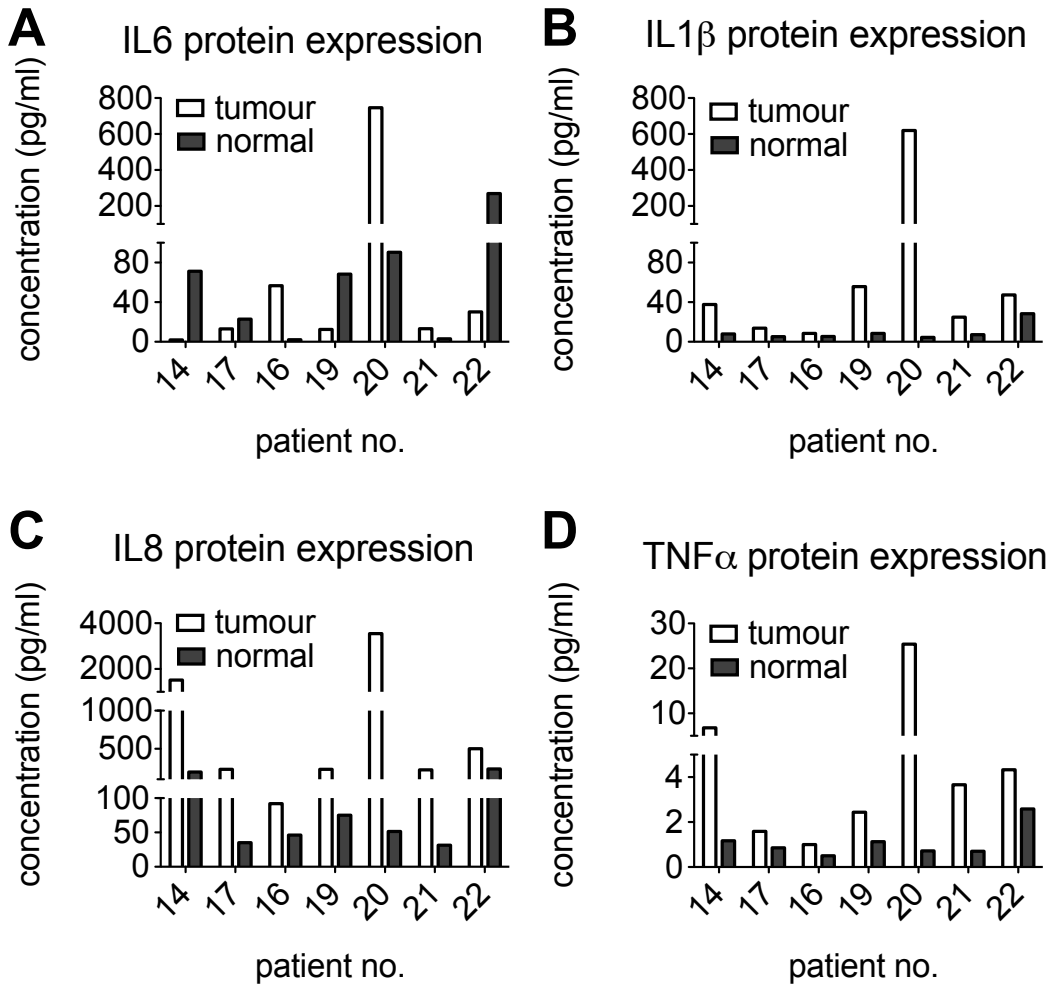

Figure S.6 Pro-inflammatory cytokine protein expression expression in CRC tissue samples in individual patients. Cytokine concentration was measured in protein extracted from tissue samples using the MSD platform technology. Average concentration (pg/ml) is plotted for tumour and adjacent normal tissue samples for each patient individually (n=7).

Appendix C: Cellular behavioural changes in 2D *vs* 3D cell culture

Tumours are spheres of cancer cells with a hypoxic core and a dynamic 3D microenvironment, which is thought to play an essential role in tumour biology. Standard *in vitro* methods consist of growing cells on flat plastic surfaces and hence fail to accurately replicate this 3D environment. Increasingly, novel culture methods are being developed in which cancer cells are cultured as 3D spheroids, thus recreating the *in vivo* tumour architecture in an *in vitro* environment. In this project, I used 3D cell culture to validate findings from 2D culture models. In order to determine if this new culture environment changes cellular behaviour, I performed various experiments comparing cells grown in these two different culture systems.

Cells cultured in 3D bioscaffolds were shown to have decreased cell growth (Figure S.6) and migration (Figure S.7), while cell invasion was increased compared to 2D cultures (Figure S.7). Dietary carcinogen-induced micronuclei formation was increased in 3D culture (Figure S.8). While corresponding increase in *CYP1B1* gene expression was observed, *CYP1A1* gene expression was decreased in 3D compared to 2D culture (Figure S.9). However, EROD activity was increased in 3D cells (Figure 2.13 B), which could result in increased activation of the dietary carcinogens in 3D culture, thus possibly explaining why increased DNA damage was observed. Differences in gene and miRNA expression were observed between both cell culture systems (Figure S.9 and S.10) including downregulation of *E-cadherin* and *p53*, and upregulation of miR125b and miR31 in 3D cultures. This expression pattern is commonly found

in colorectal tumours *in vivo* (Slaby *et al.* 2009) and known to be involved in cancer progression.

Taken together, these data suggest that cells grown as 3D spheroids have altered cellular behaviour compared to cells grown as monolayers and these differences are likely to be driven by changes in the genetic and epigenetic make-up of the cells.

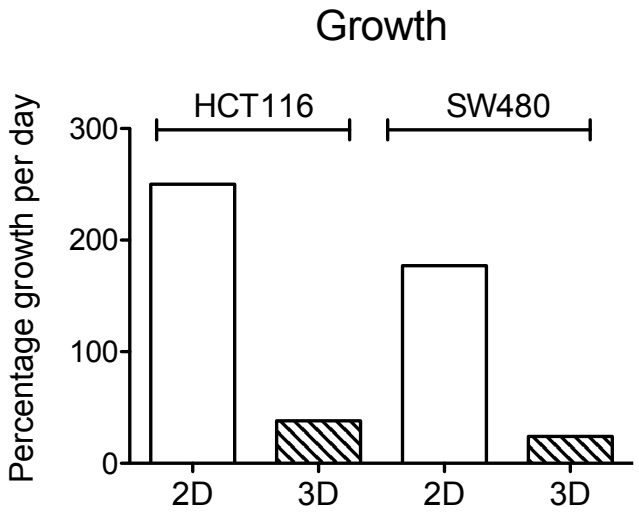

Figure S.7 Cell growth of HCT116 and SW480 cultured as 2D monolayers and 3D spheroids. Cells were grown on as monolayers or as 3D spheroids. Cell growth was determined by counting the number of cells using a haemocytometer with Trypan Blue exclusion. Data are expressed as average percentage growth per day (n=1).

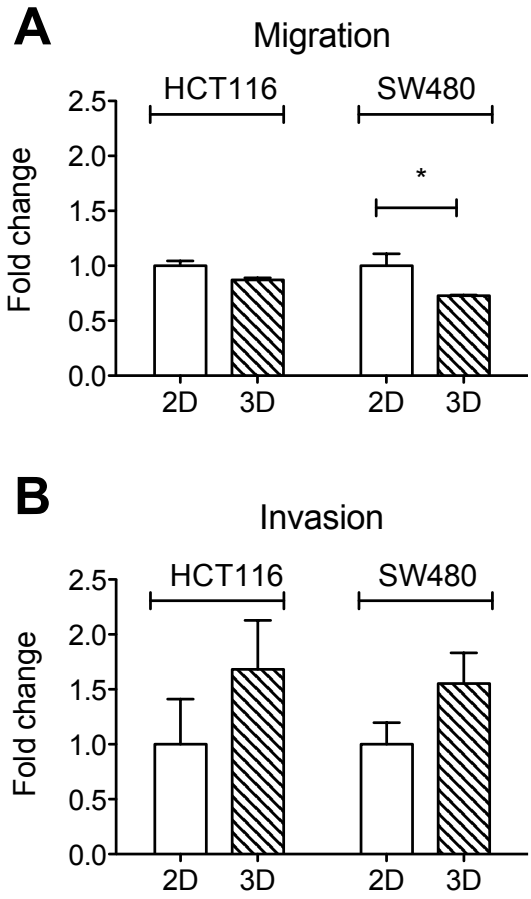

Figure S.8 Migration and invasion of HCT116 and SW480 cells grown as 2D monolayers and 3D spheroids. Cells were grown on as monolayers or as 3D spheroids. Cells/ spheroids were added to the upper chamber. The transwell migration (A) and invasion (B) assays were carried out over 72 hours. 10% FBS was added to the bottom chamber as a chemoattractant. For the invasion assay, the upper chamber coated with matrigel prior to adding the cells. Cells appearing in the bottom compartment were quantified using AlamarBlue. Data are expressed as fold change compared to 2D. Significance was calculated using Student's *t*-test (GraphPad Prism 5, $*p<0.05$). Error bars represent the SEM for independent cultures (n=3).

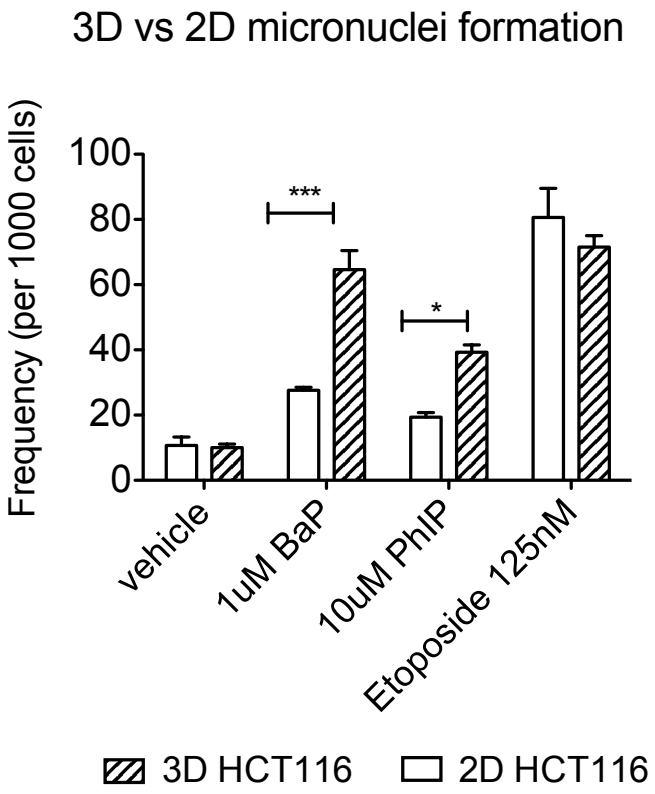

Figure S.9 Micronuclei frequency in HCT116 grown as 2D monolayers and 3D spheroids. Cells grown as monolayers and 3D spheroids were treated for 24 hour with BaP or PhIP; cells were taken 72 hours post-treatment. Etoposide was used as a positive control. Micronuclei frequency per 1000 cells was measured following treatment. Statistically significant differences are shown for comparisons between cells grown in 2D or 3D culture (***p<0.001, **p<0.01, *p<0.05). Significance was calculated using one-way ANOVA with a Dunnett post-test (GraphPad Prism 5). Error bars represent the SEM for independent cultures (n=3).

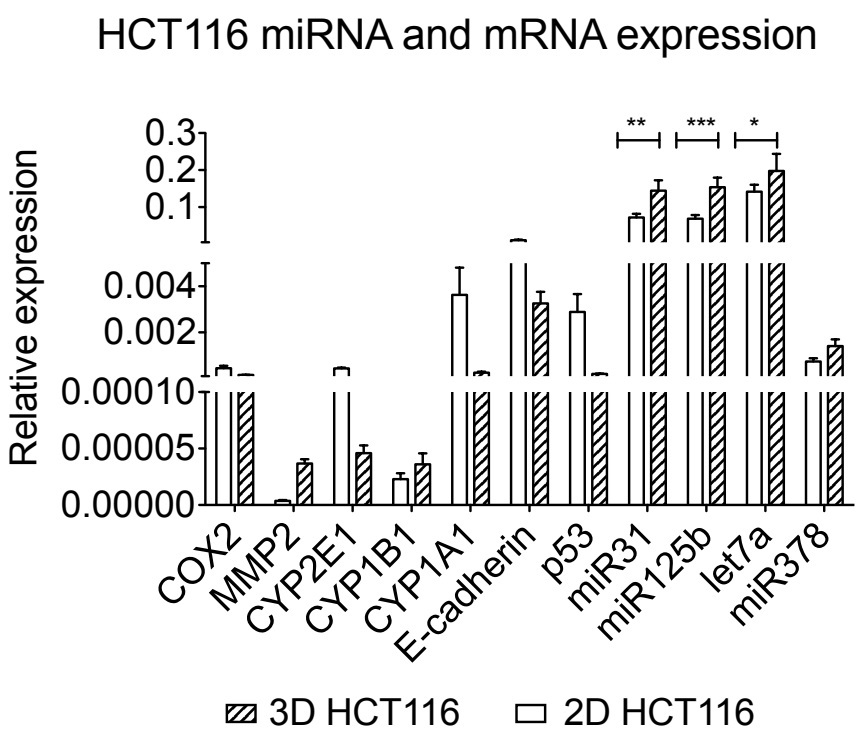

Figure S.10 Comparison of gene and miRNA expression in HCT116 grown as 2D monolayers and 3D spheroids. Cells were grown as monolayers and 3D spheroids. Gene and miRNA expression were measured by RT-qPCR. Data were normalised to expression of *GAPDH* and U6 RNA for gene and miRNA expression respectively; data are presented as relative expression. Significance was calculated using two-way ANOVA with a Bonferroni post-test comparing 2D to 3D cell culture (GraphPad Prism 5, *p<0.05, **p<0.01). Data are presented as a mean of three biological replicates. Error bars represent the SEM for independent cultures.

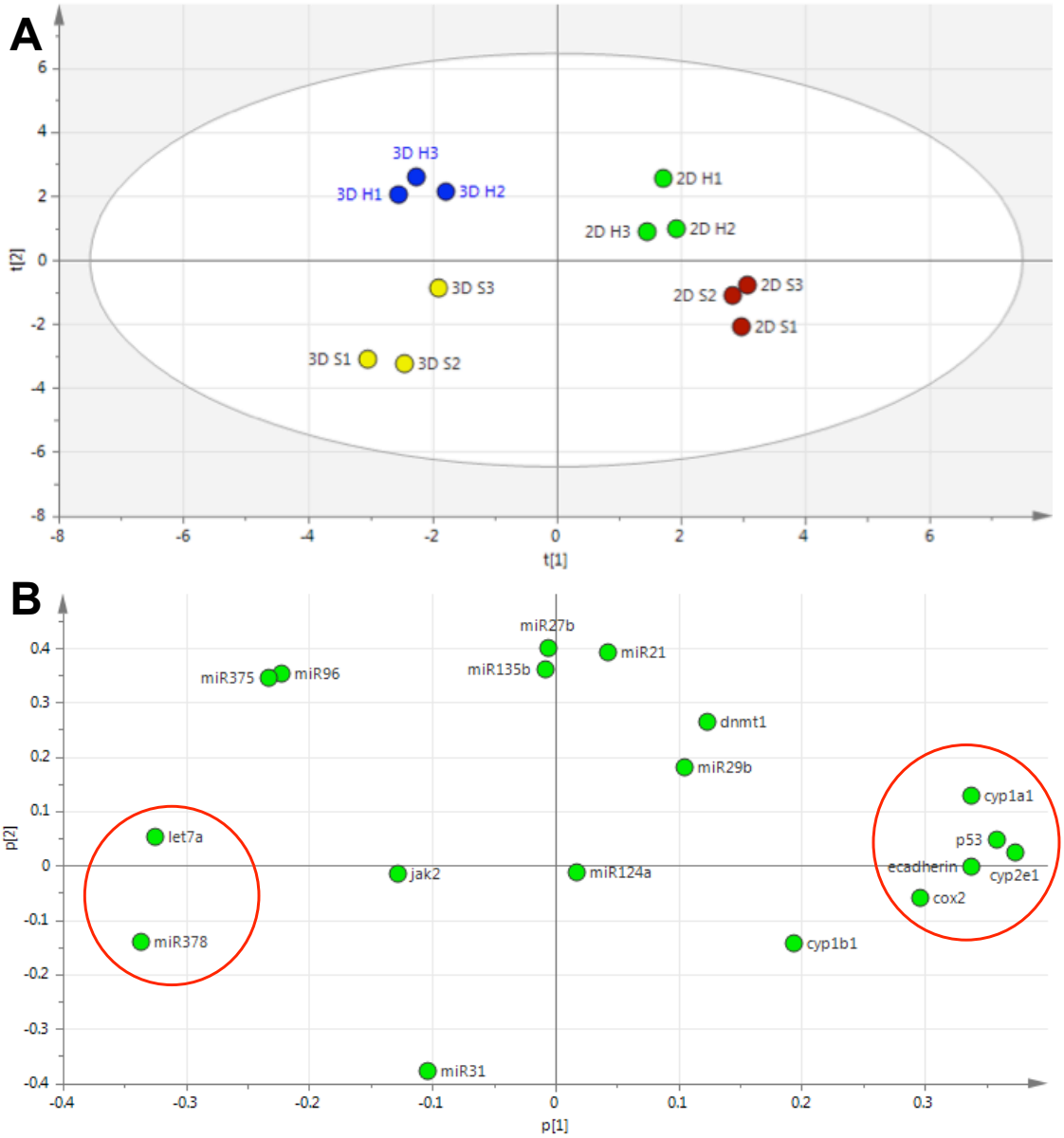

Figure S.11 Principle component analysis of gene and miRNA expression in HCT116 and SW480 grown as 2D monolayers and 3D spheroids. Cells were grown as monolayers and 3D spheroids. Gene and miRNA expression were measured by RT-qPCR. Data were normalised to expression of *GAPDH* and U6 RNA for gene and miRNA expression respectively; relative expression was used in the principle component analysis (SIMCA, Umetrics, Crewe, UK). Scatter-plot with the two first largest components (A) and associated loading-plot with the gene and miRNAs responsible for the 2D *vs* 3D separation circled in red (B). Colours indicate the different classes in the data: 3D SW480 in yellow, 2D SW480 in red, 3D HCT116 in blue and 2D HCT116 in green (each data point represents a biological replicate).

Appendix D: Published research article

Patel, S.A.A. *et al.*, 2014. Interleukin-6 mediated upregulation of CYP1B1 and CYP2E1 in colorectal cancer involves DNA methylation, miR27b and STAT3. *British journal of cancer*, 111(12), pp.2287-96 (attached on the next page).

Patel, S.A.A. and Gooderham, N.J., 2015. Interleukin-6 promotes dietary carcinogen-induced DNA damage in colorectal cancer cells. *Toxicology Research*, 4, pp.858-66 (attached on page 228).

FULL PAPER

BJC

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Keywords: inflammation; microRNA; cytochrome P450; colorectal cancer; immunohistochemistry

Interleukin-6 mediated upregulation of CYP1B1 and CYP2E1 in colorectal cancer involves DNA methylation, miR27b and STAT3

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Background: The pro-inflammatory cytokine interleukin-6 (IL6) promotes colorectal cancer (CRC) development. It is also known to regulate cytochrome P450 (CYP450) enzymes, which are involved in CRC tumour initiation and promotion via activation of chemical carcinogens. Here, IL6 regulation of CYP450 expression was investigated in CRC.

Methods: The effect of IL6 on CYP 1A1, 1B1 and 2E1 expression was determined *in vitro* using CRC cell lines HCT116 and SW480, and CYP450 expression was determined by immunohistochemistry in CRC tissues previously shown to have increased levels of IL6.

Results: In mechanistic studies, IL6 treatment significantly induced *CYP1B1* and *CYP2E1*, but not *CYP1A1*, gene expression in HCT116 and SW480 cells. *CYP2E1* expression regulation occurred *via* a transcriptional mechanism involving STAT3. For *CYP1B1* regulation, IL6 downregulated the CYP1B1-targeting microRNA miR27b through a mechanism involving DNA methylation. In clinical samples, the expression of CYP1B1 and CYP2E1, but not CYP1A1, was significantly increased in malignant tissue overexpressing IL6 compared with matched adjacent normal tissue.

Conclusions: Colonic inflammation with the presence of IL6 associated with neoplastic tissue can alter metabolic competency of epithelial cells by manipulating *CYP2E1* and *CYP1B1* expression through transcriptional and epigenetic mechanisms. This can lead to increased activation of dietary carcinogens and DNA damage, thus promoting colorectal carcinogenesis.

Colorectal cancer (CRC) is one of the most common malignancies in the Western world where it is the second highest cause of cancer-related mortality (Tenesa and Dunlop, 2009). Despite multiple advances in treating the disease, 5-year survival rates have not significantly improved in the last 20 years highlighting the need for new diagnosis strategies.

Local inflammation in the colon is a known risk for CRC development and progression (Feagins *et al*, 2009). We have previously reported increased expression of a number of inflammatory markers including pro-inflammatory cytokine interleukin-6 (IL6), a key regulator of inflammation, in colon tumour tissue and stroma (Figure 1) (Maihofner *et al*, 2003; Charalambous *et al*, 2003; Charalambous *et al*, 2009). Indeed, stromal cells including tumour-associated immune cells and fibroblasts release high levels of IL6 (up to 6000 pg ml^{-1}) in the colorectal tumour microenvironment (Nagasaki *et al*, 2014). Furthermore, elevated serum levels of IL6 are associated with poor disease outcome and non-steroidal anti-inflammatory drugs are known to reduce CRC risk by lowering levels of inflammation (Williams *et al*, 1997; Smalley *et al*, 1999).

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Appendix D

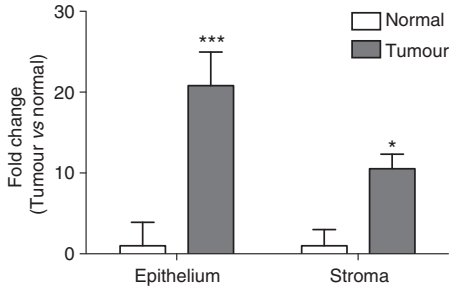

Figure 1. IL6 expression is increased in epithelial cells and stroma of colorectal tumour samples. Data are adapted from our previous publication (Maihofner *et al*, 2003) and are shown as fold change of immunohistochemistry score relative to control. Significant differences from normal tissue were calculated using Wilcoxon's signed-rank test (*$P<0.05$, ***$P<0.001$).

Interleukin-6 is also known to regulate CYP enzyme expression. Studies in hepatocytes have generally shown that IL6 inhibits CYP450 expression including CYP 1A1, 1A2, 3A4 and 2E1 (Abdel-Razzak *et al*, 1993; Jover *et al*, 2002; Hakkola *et al*, 2003). However, conflicting results have been reported; IL6 has also been shown to induce CYP2E1 in astrocytes (Tindberg *et al*, 1996), suggesting that the effects of IL6 on CYP450 expression vary depending on tissue type and the relation between IL6 and CYP450 expression has not yet been defined in the colon. CYP1B1 is known to be overexpressed in a number of tumour types including lung, colon and breast (Murray *et al*, 1997). A recent study in human hepatocellular carcinoma cells has reported that IL6 treatment induced CYP1B1 gene and protein expression (Kurzawski *et al*, 2012), suggesting that IL6 is able to regulate CYP1B1. However, there have been very few other studies to confirm this effect of IL6 on CYP1B1. Furthermore, despite these numerous reports of IL6 regulating CYP450s, the specific molecular mechanisms underlying the effects are not yet well understood.

MicroRNAs (miRNAs) are small, ~22 nucleotide long non-coding gene-regulating RNAs; they silence gene expression by binding to target mRNAs. MicroRNAs only require partial base complementarity to bind to their targets and can thus target a number of different mRNAs. Recent studies have found that miRNAs can modulate CYP450 expression. For instance, miR27b and miR378 have been reported to directly regulate CYP1B1 and CYP2E1 expression, respectively (Tsuchiya *et al*, 2006; Mohri *et al*, 2010). Furthermore, emerging evidence suggests that epigenetic events including DNA methylation and miRNA expression are involved in CRC initiation and progression (Suzuki *et al*, 2012). Interestingly, IL6 has been shown to alter miRNA expression profiles (Yang *et al*, 2010) and to induce genome-wide methylation of promoter regions (Webbe *et al*, 2006) resulting in gene silencing, thus epigenetic mechanisms may be involved in IL6-mediated regulation of CYP450s.

To investigate the relationship between IL6 and CYP450 expression in the neoplastic colon, we first determined the expression of the CYP450 enzymes CYP 1A1, 1B1 and 2E1 in the human CRC cell lines HCT116 and SW480 with subsequent mechanistic studies to confirm increased levels of IL6 could alter CYP450 expression and propose underlying mechanisms. Here to extend the *in vitro* results, we measured the expression of the same CYP450s in malignant tissues resected from CRC patients that have increased expression of IL6 in the epithelium and stroma (Figure 1; Maihofner *et al*, 2003), and compared expression with that in adjacent normal tissues.

MATERIALS AND METHODS

Cell culture. The human colorectal adenocarcinoma cell lines HCT116 and SW480 were obtained from ATCC (LGC Prochem, Middlesex, UK) and were routinely cultured in RPMI-1640 medium (GIBCO, Life Technologies, Paisley, UK) supplemented with 10% fetal bovine serum (FBS), 100 units ml^{-1} penicillin, 100 $\mu g\,ml^{-1}$ streptomycin and 2 mM L-glutamine (GIBCO, Life Technologies). All cells were incubated at 37 °C in a humidified incubator (5% CO_2). Cells between passages 3 and 7 were used in the experiments.

IL6 treatment and demethylation/STAT3 inhibition. Before treatment, HCT116 and SW480 were maintained in culture medium supplemented with 5% dextran-coated charcoal-stripped FBS for at least 72 h. Cells were seeded at a density of 1×10^5 cells per well of a 6-well plate. HCT116 and SW480 do not express IL6; cells were treated for 24 and 48 h with recombinant human IL6 (HumanKine, Sigma-Aldrich, Dorset, UK) dissolved in phosphate-buffered saline (PBS) containing 0.1% human serum albumin (Sigma-Aldrich) at doses of 0, 100 and 1000 pg ml^{-1} (chosen within the range secreted by stromal cells in colon tumour microenvironment). For demethylation/STAT3 inhibition, cells were co-treated for 24 and/or 48 h with 1000 pg ml^{-1} IL6 and either 4 μM 5-aza-2′-deoxycytidine (Sigma-Aldrich) or 25 μM STAT3 inhibitor (STAT3 inhibitor VIII 5,15-diphenylporphyrin, Millipore, Feltham, UK), respectively. STAT3 inhibitor and 5-aza-2′-deoxycytidine were dissolved in DMSO (final vehicle control concentration of 0.1%).

RNA extraction. Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Life Technologies) according to the manufacturer's protocol. RNA extracts were quantified by UV spectroscopy (UV–vis Spectrophotometer, Implen, Essex, UK) with purity assessed from 260/280 nm and 260/230 nm ratios. Extracts were stored at −80 °C until used.

Reverse transcription and quantitative PCR. For mRNA reverse transcription, RNA extracts (100–500 ng) from each sample were added to 300 ng of random primers, heated for 5 min at 65 °C and then immediately placed on ice. Each sample was incubated with 0.5 mM dNTPs, 1 × first strand buffer, 8 μM dithiothreitol and 100 units of Superscript II reverse transcriptase (Invitrogen, Paisley, UK) for 10 min at 25 °C, 90 min at 42 °C and 15 min at 70 °C on a thermocycler (Peltier Thermal Cycler PTC-200, MJ Research, Waltham, MA, USA). An miRNA reverse transcription kit was used for miRNA expression according to the manufacturer's protocol (Taqman, Applied Biosystems, Life Technologies, Paisley, UK). Quantitative PCR was performed using pre-designed gene expression assays and FAST PCR master mix (Taqman, Applied Biosystems, Life Technologies), and measured in a StepOnePlus fast real-time PCR system (Applied Biosystems, Life Technologies) according to the manufacturer's protocol. Gene expression was quantified using the delta-C_t method.

Chromatin immunoprecipitation-quantitative PCR. SW480 cells were treated with 1000 pg ml^{-1} IL6 and 25 μM STAT3 inhibitor for 60 min before collecting the cells. Chromatin immunoprecipitation (ChIP) assay was performed using a magnetic ChIP kit (Thermo Fisher Scientific, Cramlington, UK) as per manufacturer's protocol with an anti-STAT3 antibody (sc-482X, Santa Cruz Biotechnology, Heidelberg, Germany). Rabbit IgG was used as a negative control for non-specific binding. Binding was calculated as a percentage of the total input chromatin. Sequences of the primers used were as follows:

CYP2E1 site 1 forward: 5′-TGAATTTTCCTTCTGGCCCCAT-3′, reverse 5′-TGATGAGGAGGTTTGTCTGAGC-3′;
CYP2E1 site 2 forward: 5′-CTCCATCCTCACCAGGTCAC-3′, reverse: 5′-CCAACCAATGCCCTCTTGCT-3′.

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Patients. The study adhered to the tenets of the Declaration of Helsinki. Surgical specimens of primary tumours and adjacent normal colon were obtained with informed consent from 40 patients (28 men and 12 women; aged 49–80 years, mean age 64.7 years ± 8.1 s.e.m.), with histologically verified colorectal cancer, treated at the Department of Surgery, York District Hospital, York, UK. Ethical approval for the study was obtained from the Human Research Ethics Committee at York District Hospital. Nineteen patients had colon cancer and twenty-one rectal cancers. Tumours were classified according to Duke's classification (Table 1). The entire study was carried out blind using coded tissue sections. Eligibility criteria for patients recruited to the study included Caucasian origin, 45–80 years of age and no history of previous gastrointestinal disease or any form of cancer, including familial adenomatous polyposis.

Tissue specimens. Tissue samples taken at operation for histopathological confirmation of disease were fixed in 4% buffered formaldehyde and embedded in paraffin wax; sections surplus to pathology requirements were made available for the study. For all 40 patients, tissue sections of both malignant and normal (taken outside the tumour margin) colon or rectum were provided.

Antibodies and reagents. Specific anti-peptide antibodies raised against individual CYP450 enzymes and the corresponding pre-immune sera have been previously described (Edwards *et al*, 1998; Kapucuoglu *et al*, 2003). These antibodies have previously been used to detect human CYP450 protein expression (Piipari *et al*, 2000, Rodriguez-Antona *et al*, 2002). Biotin labelled secondary antibodies and streptavidin/HRP complex were obtained from DAKO Ltd (Ely, UK). All other chemicals were supplied by either Sigma-Aldrich or Merck (Nottingham, UK) unless otherwise specified.

Immunohistochemistry. The levels of expression and localisation of CYPs 1A1, 1B1 and 2E1 in malignant and adjacent normal colorectal tissue were determined using a modified avidin/biotin immunohistochemistry procedure (Goggi *et al*, 1986). In preliminary experiments, each of the immunohistochemistry assays was optimised using a range of antisera dilutions (1/200 to 1/8000). For each assay, the negative control antisera (pre-immune sera) were confirmed negative for staining at the dilution optimised for the primary antibody and blocking peptides confirmed specificity. The dilutions used were 1/5000 for the anti-CYP1A1 antibody, and 1/1000 for the anti-CYP1B1 and anti-CYP2E1 antibodies. The sections were deparaffinised and rehydrated through xylene and a series of graded alcohol solutions. Endogenous peroxidase activity was blocked by immersing the sections into a solution of 3% hydrogen peroxide in distilled water for 30 min at room temperature, and then rinsed in cold running tap water for 10 min. Incubating the sections with 5% normal swine serum for 30 min at room temperature reduced non-specific background staining. Sections were then washed twice with PBS and either the primary antibody or the normal goat or rabbit IgGs (negative control) was applied to each section and left at 4 °C overnight. The next day, the slides were washed twice with PBS, and then incubated with the secondary antibody solution (Biotinylated swine anti-goat, mouse, rabbit immunoglobulin; 1/150 dilution), for 1 h at room temperature. After being washed twice with PBS, they were incubated with the StrepABComplex solution for 1 h at room temperature, washed twice with PBS and immersed into the substrate (300 ml PBS, 90 μl hydrogen peroxide and 2.5 ml 3,3-diaminobenzidine) for 3 min, and then rinsed with PBS and cold running tap water. Sections were then successively immersed into haematoxylin, acid alcohol and Scott's tap water to counterstain. Finally, the sections were dehydrated by successive immersion into 70% ethanol, 100% ethanol twice and xylene twice and mounted.

Immunohistochemical evaluation. Processed specimens were scored under the light microscope and the intensity and localisation of staining with CYPs 1A1, 1B1 and 2E1 antibodies graded blind using coded slides. To assess and grade intensity and distribution of immunoreactivity in the epithelial cells, a previously described method of scoring the sections was used (Maihofner *et al*, 2003, Charalambous *et al*, 2003, 2009). The distribution was scored according to the number of positive cells; none (not stained), 0; focal (<1/3 of cells stained), 1; multi-focal (1/3 to 2/3 of cells stained), 2; and diffuse (>2/3 stained), 3; staining intensity was scored as: none (not stained), 0; lightly stained, 1; and strong staining, 2. The distribution and intensity scores were added to produce the grade of staining. Sections treated with the normal goat or rabbit IgGs (negative controls) or omitting the primary antibody were devoid of staining. Positive staining controls included sections of kidney, duodenum and liver.

Statistical analysis. The Wilcoxon's signed-rank test was used to compare the scoring of the respective immunoreactivity for CYP 1A1, 1B1 and 2E1 between tumour and adjacent normal tissues (Stata Statistical Software 9, StataCorp LP, College Station, TX, USA). Gene expression data were obtained from measurements made in at least three biological replicates and presented as a mean ± standard error. Significant differences ($P<0.05$) were determined using Student's *t*-test and one-way analysis of variance (ANOVA) followed by a Dunnett post-test or a linear trend analysis (GraphPad Prism 5, GraphPad Software Inc., La Jolla, CA, USA). Pearson's product-moment correlation coefficient test was used for correlation analysis (GraphPad Prism 5).

RESULTS

Can IL6 regulate CYP450 expression in CRC cells? To determine whether IL6 can regulate CYP450s in colon cancer cells, we performed mechanistic studies in *in vitro* models (human CRC cell lines HCT116 and SW480) to examine the effect of IL6 treatment on *CYP 1A1*, *1B1* and *2E1* gene expression at various time points using quantitative PCR.

CYP1A1 gene expression was detected but not significantly changed in either cell line following 24- and 48-h IL6 treatment (Figure 2A). However, *CYP1B1* and *CYP2E1* mRNA expression was regulated dose dependently by IL6 as determined by positive trend analyses and was significantly increased at the highest dose of 1000 pg ml^{-1} IL6 in both cell lines (Figure 2B and C). To the best of our knowledge, this is the first account of *CYP1B1* and *CYP2E1* being upregulated by IL6 in colon tumour-derived epithelial cells.

What are the mechanisms involved in IL6-mediated upregulation of CYP2E1 and CYP1B1?

IL6 regulates CYP2E1 expression through STAT3 transcription factor. To understand the mechanism underlying IL6 induction of *CYP2E1* expression, we examined the different pathways involved in *CYP2E1* regulation. CYP2E1 is regulated at various stages of its synthesis and includes transcriptional and post-transcriptional mechanisms. We looked at miRNA-mediated regulation of *CYP2E1* mRNA by determining miR378 expression, a miRNA reported to target *CYP2E1* (Mohri *et al*, 2010). However, no change in miR378 expression by IL6 was observed (Figure 3A) and we found no correlation between *CYP2E1* and miR378 expression in our model (Figure 3B).

IL6 is a potent inducer of the JAK/STAT3 pathway. An analysis of the *CYP2E1* promoter region revealed multiple potential STAT binding sites (Figure 4A; TFSEARCH ver1.3; Heinemeyer *et al*, 1998). We thus studied involvement of STAT3 in regulating IL6-mediated *CYP2E1* induction using a STAT3 inhibitor, STAT3 inhibitor VIII 5,15-diphenylporphyrin. Treatment with the inhibitor prevented IL6-mediated *CYP2E1* induction after 24 h in both HCT116 and SW480

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Table 1. Patient demographic information

Patient	Age[a]	Sex	Tumour site	Dukes' stage	Drug history	Tobacco use	Alcohol[b]
1	59	M	Rectum	A	None	No	1–7
2	58	F	Colon	B	Azathioprine, Insulin, Prednisolone, Lisinopril, Frusemide, Ferrous sulphate	No	1–7
3	79	M	Rectum	B	Atenolol, Bendrofluazide	No	8+
4	72	M	Colon	B	None	No	8+
5	60	F	Rectum	B	Lipostat	No	8+
6	66	F	Colon	C	5-Fluorouracil, Enalapril	No	0
7	69	M	Rectum	C	Co-codamol	No	1–7
8	52	M	Rectum	C	Adalat	No	1–7
9	68	M	Colon	B	Atenolol, Prednisolone, Warfarin, Ferrous sulphate, Diltiazem, Isosobrbite mononitrate, Gliclazide, Co-danthramer	No	1–7
10	69	M	Rectum	C	Atenolol	No	8+
11	70	F	Rectum	B	Lithium, Levothyroxine	No	8+
12	72	M	Rectum	B	Captopril, Naproxen, Allopurinol, Isosobrbite mononitrate, Frusemide, Atenolol, Prochlorperazine	No	1–7
13	56	M	Colon	A	None	No	8+
14	76	M	Colon	A	None	No	1–7
15	58	F	Colon	N/K	None	No	0
16	66	F	Colon	B	None	No	0
17	54	M	Rectum	C	None	No	1–7
18	49	M	Colon	B	None	No	8+
19	52	M	Rectum	B	None	No	8+
20	68	M	Colon	B	Salbutamol, Ferrous sulphate	No	0
21	63	F	Rectum	A	Salbutamol, Beclomethasone, Bendrofluazide	No	1–7
22	56	M	Colon	C	Losec	No	8+
23	68	M	Rectum	B	Sotalol, Aspirin	No	8+
24	80	F	Rectum	B	None	No	0
25	59	M	Rectum	C	None	No	8+
26	66	M	Rectum	N/K	None	Current	8+
27	59	F	Colon	N/K	Fibrogel	No	0
28	74	M	Colon	N/K	None	No	1–7
29	64	M	Rectum	B	Aspirin, Omeprazole, Simvastatin	No	0
30	72	F	Rectum	C	None	No	8+
31	50	M	Rectum	B	Betagan	Current	1–7
32	72	M	Rectum	B	Diclofenac, Propanolol, Adalat, GTN, Isosorbide mononitrate	No	8+
33	55	M	Colon	B	None	No	1–7
34	67	F	Rectum	C	Ibuprofen, Aspirin, Bendrofluazide	No	1–7
35	74	M	Colon	C	5-Fluorouracil	No	8+
36	73	M	Colon	A	None	No	8+
37	60	M	Colon	C	None	No	1–7
38	59	F	Rectum	B	Voltarol	No	0
39	73	M	Colon	C	5-Fluorouracil, Co-danthrusate, Manevac	No	1–7
40	70	M	Colon	C	Ferrous sulphate	No	0

Abbreviation: N/K = not known.
[a]Age in years.
[b]Alcohol consumption in units per week (1 unit = half a pint of beer or one glass of wine or one shot of spirits).

cell lines (Figure 4B). Furthermore, a ChIP analysis in SW480 cells revealed that STAT3 does bind to the CYP2E1 promoter region following IL-6 treatment (Figure 4D), compatible with a STAT3-mediated mechanism for induction of *CYP2E1* expression by IL6.

IL6 regulates CYP1B1 expression through repression of miR27b. We next examined the mechanisms underlying IL6-mediated induction of *CYP1B1* expression. The aryl hydrocarbon receptor (AhR) pathway is a well-known transcriptional regulator of *CYP1B1* and *CYP1A1* expression. However, *CYP1A1* mRNA expression was not induced upon IL6 treatment (Figure 2A), thus the AhR pathway is unlikely to be involved in IL6-mediated induction of *CYP1B1*. Furthermore, we determined that STAT3 was not involved in regulating IL6-mediated *CYP1B1* induction, as treatment with the STAT3 inhibitor did not affect IL6-mediated induction of CYP1B1 (Figure 4C).

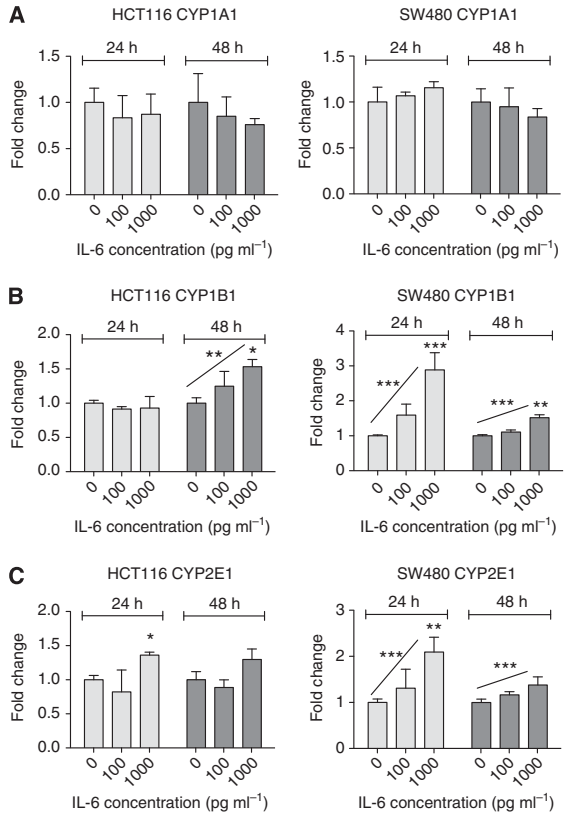

Figure 2. IL6 effect on *CYP450* gene expression. HCT116 and SW480 cells were treated with 0, 100 and 1000 pg ml^{-1} IL6 for 24 and 48 h. *CYP1A1* (**A**), *CYP1B1* (**B**) and *CYP2E1* (**C**) expression was measured by RT-qPCR. Data were normalised to expression of *GAPDH* housekeeping gene and are shown relative to control. Significance was calculated using one-way ANOVA with a Dunnett post-test comparing treated groups with vehicle control and linear trend analysis (*$P<0.05$, **$P<0.01$, ***$P<0.001$). Data are presented as a mean of at least three biological replicates. Error bars represent the s.e.m.

MiR27b has been reported to directly target *CYP1B1* mRNA by binding to its 3'UTR to regulate its expression (Tsuchiya *et al*, 2006). IL6 significantly downregulated miR27b expression in both cell lines in the current study (Figure 3C). Furthermore, there was a significant inverse correlation between miR27b expression and *CYP1B1* expression (Figure 3D), suggesting that downregulation of miR27b could be responsible for the increase in *CYP1B1* mRNA observed. To our knowledge, this is the first account of IL6 modulating miR27b expression, thus providing a potential post-transcriptional mechanism by which *CYP1B1* is regulated by IL6.

How does IL6 cause miR27b downregulation? We next determined the mechanism underlying miR27b regulation by IL6. MiR27b is an intragenic miRNA located within the *C9orf3* gene on chromosome 9. Previous reports have shown that an intragenic CpG island (chr9: 96 887 100–96 887 300) located close to miR27b can be methylated in colon cancer cells and regulates miR27b expression (Yan *et al*, 2011). Interleukin-6 is known to induce genome-wide DNA methylation through phosphorylation of DNMT1 by activated AKT, which increases DNMT1 nuclear translocation and activity (Hodge *et al*, 2007); therefore, we examined the role of DNA methylation in IL6 regulation of miR27b expression by inhibiting DNA methylation using 5-aza-2′-deoxycytidine treatment. We observed that by inhibiting DNA methylation, we were able to prevent miR27b downregulation by IL6 in both cell lines (Figure 5A), implying that DNA methylation has a role in regulating miR27b expression. Again, these changes inversely correlated with *CYP1B1* expression (Figure 5B and C). These mechanistic data suggest that IL6-mediated upregulation of CYP1B1 involves downregulation of miR27b via DNA methylation.

Is CYP450 expression altered in malignant colorectal tissues that overexpress IL6? Having shown that IL-6 regulates expression of CYP1B1 and 2E1, but did not alter expression of CYP1A1 in CRC cells, to confirm whether this may be relevant *in vivo,* we measured the expression of these CYP450s by immunohistochemistry (Figure 6) in tissue sections of malignant and normal bowel from colorectal cancer patients, in which IL6 expression has previously been determined (Figure 1; Maihofner *et al*, 2003).

In most cases, we observed immunostaining only in epithelial cells. Expression of CYP was generally greatest in luminal enterocytes with involvement of the upper third of the crypts in some individuals. We did not observe any expression in the base of crypts. Within the cells, we noted the expression was cytoplasmic with the intensity of expression greatest in perinuclear regions, suggesting localisation within the endoplasmic reticulum. No expression was observed in the mucous inclusions of goblet cells. In tumour tissue, staining was confined to epithelial cells and there was no significant intratumour heterogeneity in enzyme expression.

The CYP1A1 expression was observed in epithelial cells of normal colorectal tissue in 7 out of 40 patients, while 11 out of 40 patients expressed CYP1A1 in malignant epithelial cells (Figure 6C and D). Using our previously described immunohistochemistry scoring procedures (Maihofner *et al*, 2003; Charalambous *et al*, 2003, 2009), we found that this did not translate into a significant difference (Figure 6K). Expression of CYP1B1 in normal colorectal tissue was detected in 18 out of 40 patients (Figure 6E), while the majority of patients expressed CYP1B1 in malignant tissue (35 out of 40 patients; Figure 6F). CYP1B1 was also expressed relatively intensely in histologically normal crypts that were immediately adjacent to tumour cells (Figure 6I). CYP2E1 was expressed in both normal and tumour tissues, although expression was more intense in malignant tissue (Figure 6G and H). In addition to epithelial cells, plasma cells (found in the lamina propria) also expressed CYP2E1 intensely (Figure 6J, inset).

We compared these CYP450 expression levels with patient demographics and found no significant correlation between alcohol, age, sex or tumour stage (data not shown).

More importantly, in line with our *in vitro* data, CYP1B1 and CYP2E1 expression was significantly increased in tumour samples compared with adjacent normal tissue while the CYP1A1 expression levels were not significantly changed (Figure 6K). We have previously reported increased expression of inflammatory cytokine IL6 in these same tumour tissue samples (Figure 1; Maihofner *et al*, 2003). Furthermore, *CYP1B1* and *CYP2E1* gene expression was also the only CYP450s to be induced by IL6 in our *in vitro* mechanistic models and as CYP450 mRNA levels in human cell culture have been reported to correlate well with protein expression (Rodriguez-Antona *et al*, 2002), we therefore propose that IL6 may be involved in the increased expression of these proteins in tumour tissue.

DISCUSSION

The effect of IL6 on CYP450 expression in colorectal cancer has not been widely investigated. Furthermore, only a few studies have attempted to characterise the expression of individual CYP isozymes using immunohistochemistry (Kumarakulasingham *et al*, 2005; Androutsopoulos *et al*, 2013). Certain members of the CYP450

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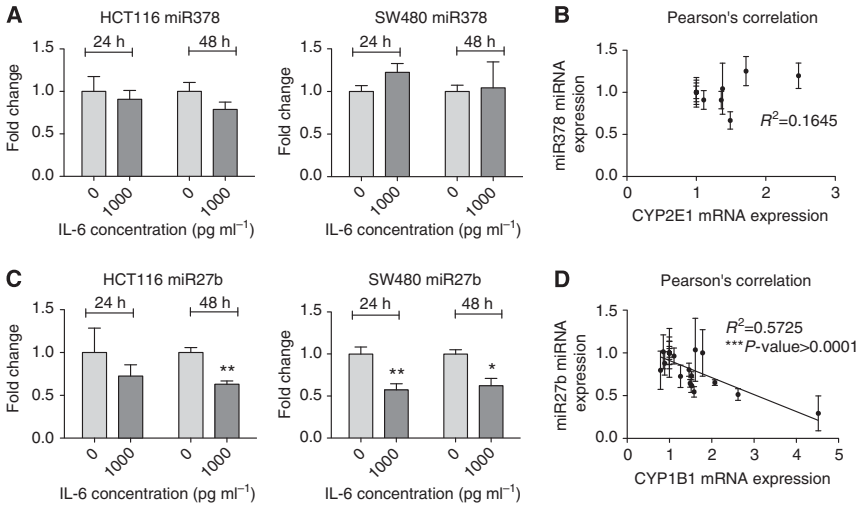

Figure 3. MiRNA involvement in IL6-mediated regulation of *CYP1B1* and *CYP2E1* gene expression. (**A**–**D**) HCT116 and SW480 cells were treated with 0 and 1000 pg ml^{-1} IL6 for 24 and 48 h. MiR378 (**A**) and miR27b (**C**) expression was measured by RT-qPCR. Fold-change expression of miR378 was correlated with fold-change expression of *CYP2E1* (**B**) and fold-change expression of miR27b with fold-change *CYP1B1* expression (**D**). Data were normalised to expression of U6 RNA and are shown relative to control. Significance was calculated using Student's *t*-test; Pearson's product-moment correlation coefficient test was used for correlation analysis (*$P<0.05$, **$P<0.01$). Data are presented as a mean of at least three biological replicates. Error bars represent the s.e.m.

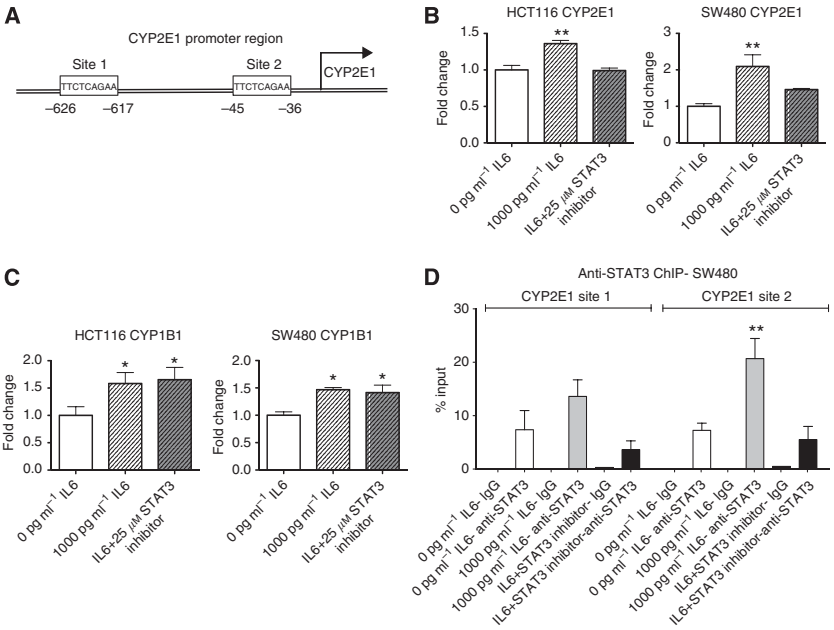

Figure 4. STAT3 involvement in IL6-mediated regulation of *CYP1B1* and *CYP2E1* gene expression. (**A**) Potential STAT3 binding sites in the CYP2E1 promoter region (1000 bp upstream of the CYP2E1 start site) predicted using TFSEARCH ver1.3 (Heinemeyer *et al*, 1998) (**B**, **C**) HCT116 and SW480 were treated with 1000 pg ml^{-1} IL6 or a combination of IL6 and 25 μM STAT3 inhibitor VII 5,15-diphenylporphyrin for 24 h (**B**) and 48 h (**C**). *CYP2E1* (**B**) and *CYP1B1* (**C**) expression was measured by RT-qPCR. Data were normalised to expression of *GAPDH* housekeeping gene and are shown relative to control. (**D**) SW480 cells were treated with 1000 pg ml^{-1} IL6 or a combination of IL6 and 25 μM STAT3 inhibitor VII 5,15-diphenylporphyrin for 60 min before being fixed and STAT3 binding to predicted sites in the CYP2E1 promoter region was measured by ChIP-qPCR using an anti-STAT3 antibody. Data are presented as a mean of at least three biological replicates for gene expression and STAT3 ChIP experiments and error bars represent the s.e.m. Significance was calculated using two-way ANOVA with a Bonferroni post-test comparing treated groups with vehicle control (**$P<0.01$). ChIP experiments were repeated using IgG antibody to control for non-specific binding and performed in duplicate.

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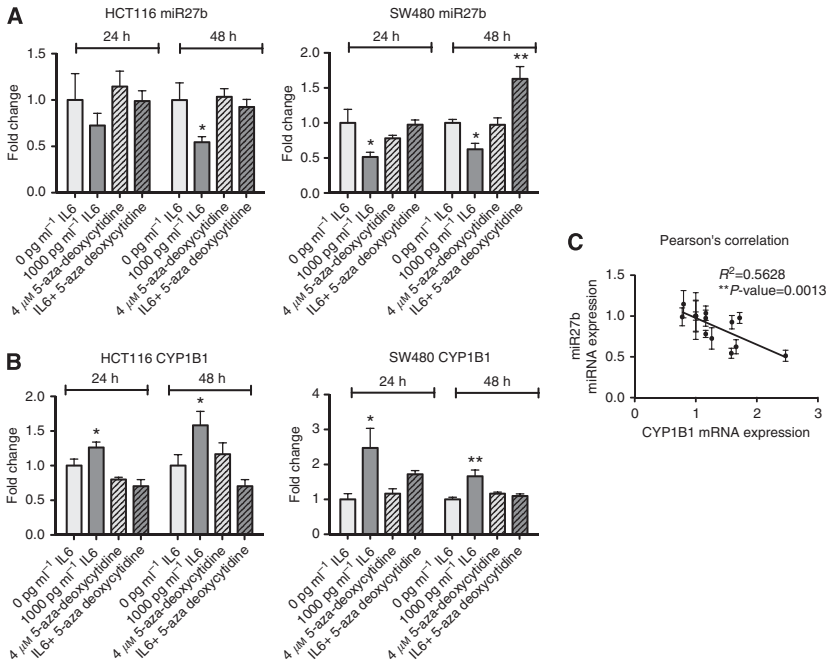

Figure 5. IL6-induced downregulation of miR27b is mediated by DNA methylation. HCT116 and SW480 cells were treated with 1000 pg ml^{-1} IL6, 4 μM 5-aza-2′-deoxycytidine or a combination of IL6 and 5-aza-2′-deoxycytidine for 24 and 48 h. (**A**) MiR27b expression was measured by RT-qPCR. Data were normalised to expression of U6 RNA and are shown relative to control. (**B**) *CYP1B1* expression was measured by RT-qPCR. Data were normalised to expression of *GAPDH* housekeeping gene and are shown relative to control. (**C**) Fold-change expression of miR27b was correlated with fold-change expression of *CYP1B1*. Significance was calculated using Student's *t*-test and one-way ANOVA with a Dunnett post-test comparing treated groups with vehicle control; Pearson's product-moment correlation coefficient test was used for correlation analysis (*$P<0.05$, **$P<0.01$). Data are presented as a mean of at least three biological replicates. Error bars represent the s.e.m.

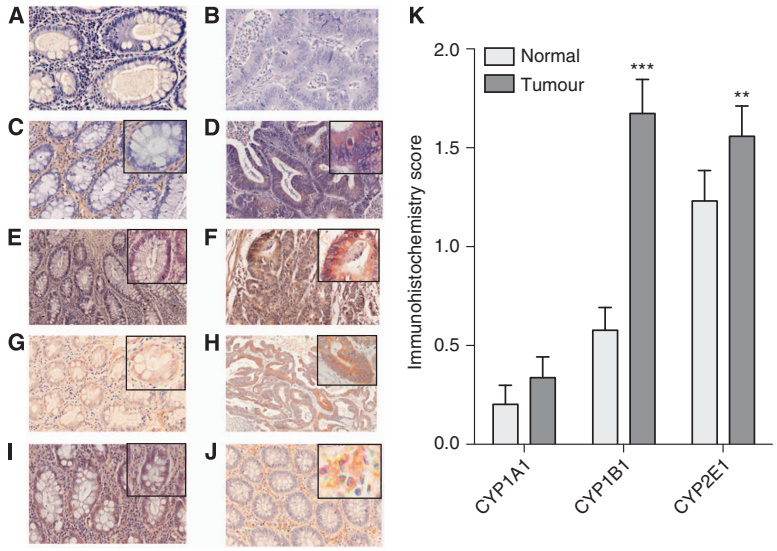

Figure 6. Immunohistochemical localisation of CYP450s in normal and adjacent malignant colonic epithelia. The presence of the immunoreactive protein is indicated by brown staining. Normal (**A**) and tumour (**B**) tissues treated with pre-immune serum as primary antibody (negative control); normal (**C**) and tumour (**D**) tissues treated with anti-CYP1A1 primary antibody; normal (**E**) and tumour (**F**) tissues treated with anti-CYP1B1 primary antibody; normal (**G**) and tumour (**H**) tissues treated with anti-CYP2E1 primary antibody. (**I**) Expression of CYP1B1 in non-malignant colonic epithelial cells lying adjacent to tumour cells. (**J**) CYP2E1 expression in plasma cells. All sections are shown at $\times 200$ or $\times 400$ (inset) magnification. (**K**) Expression of CYP450s in matched normal and adjacent malignant colonic epithelia from 40 patients. Significant differences from normal tissue were calculated using Wilcoxon's signed-rank test (**$P<0.01$, ***$P<0.001$).

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family, including CYP1A1 and 1B1, can activate dietary pro-carcinogens such as heterocyclic amines and polycyclic aromatic hydrocarbons, which have been associated with colorectal cancer (Ito *et al*, 1991; Crofts *et al*, 1997; Barrett *et al*, 2003; Boyce *et al*, 2004; Sinha *et al*, 2005; Nothlings *et al*, 2009), and the expression of CYP450 enzymes in tissues that are targets for xenobiotic genotoxicity implies a potential for *in situ* activation. CYP450s, in particular CYP2E1, can also metabolise several drugs resulting in either inhibition or activation, and thus have an important role in drug bioavailability (Koop, 1992). CYP450 enzymes have significant roles in xenobiotic activation, tumour initiation and promotion (Murray *et al*, 1997; Gooderham *et al*, 2007; Braeuning *et al*, 2011; Kasai *et al*, 2013; Rodriguez and Potter, 2013), hence, characterisation of CYP450 enzyme profiles in colon tissue and understanding the mechanisms involved in their regulation can be important for diagnosis, prognosis and treatment of disease.

Interleukin-6 has been reported to regulate CYP enzyme expression (Abdel-Razzak *et al*, 1993; Tindberg *et al*, 1996; Jover *et al*, 2002; Hokkola *et al*, 2003; Kurzawski *et al*, 2012) and is also thought to be involved in colon cancer initiation and progression (Feagins *et al*, 2009). We have previously shown IL6 expression is present at higher levels in the neoplastic colon (Maihofner *et al*, 2003) and the current study extends these observations and provides evidence for the localisation of several CYP450 enzymes in colon tissue from the same patient cohort and we demonstrate that CYP1B1 and CYP2E1 expression is significantly upregulated in these tumour tissue samples. Moreover, we have conducted mechanistic studies that give an insight into some of the regulatory mechanisms involving IL6 that underlie CYP1B1 and 2E1 enzyme expression in the colon.

Regarding CYP2E1, we found that IL6 induced *CYP2E1* expression in human colon tumour-derived cell lines and in support, we showed CYP2E1 levels were increased in tumour tissue expressing higher levels of IL6 than matched normal tissue. It is commonly thought that IL6 as well as other pro-inflammatory cytokines repress CYP450 expression (Abdel-Razzak *et al*, 1993; Jover *et al*, 2002) and *CYP2E1* gene expression was previously shown to be downregulated by IL6 in hepatic tissue (Hakkola *et al*, 2003), which is in contrast to our data. However, a study published by Tindberg *et al* (1996) reported that inflammation induced *CYP2E1* gene expression in astrocytes. Taken together, these data suggest that the effect of inflammation on *CYP2E1* expression is dependent on tissue type. We identified that IL6-mediated induction of *CYP2E1 in vitro* was through a mechanism involving direct binding of STAT3 to the *CYP2E1* promoter. This is the first account of a direct involvement of IL6 and STAT3 in *CYP2E1* expression in colonic cells. It has previously been shown that both STAT3 activation and CYP2E1 overexpression are linked to alcohol consumption (Roberts *et al*, 1995; Norkina *et al*, 2007). Epidemiological and animal studies have identified an association between excessive alcohol intake and increased risk of colorectal cancer (Pollack *et al*, 1984; Seitz *et al*, 1984; Kune *et al*, 1987; Kato *et al*, 1990). More recently, a study published by Morita *et al* (2009) demonstrated a correlation between CYP2E1 activity, alcohol intake, meat consumption and the development of colorectal cancer. Our findings provide a potential inflammatory STAT3-mediated mechanism by which CYP2E1 expression could be regulated by alcohol in the colon.

CYP1B1 has been suggested to be a tumour specific enzyme as it is rarely found in healthy tissue (Murray *et al*, 1997). As expected, there was a significant increase in CYP1B1 expression in tumour tissue when compared with matched normal tissue in the current study. These results are in agreement with previous studies, which have shown increased CYP1B1 expression in colon cancer (Murray *et al*, 1997; Gibson *et al*, 2003; Kumarakulasingham *et al*, 2005; Chang *et al*, 2005; Androutsopoulos *et al*, 2013). Moreover, in mechanistic studies we found that IL6 induced *CYP1B1* gene expression in colon tumour-derived epithelial cells. The relationship between inflammation and CYP1B1 is not as well studied as other CYP450 enzymes, despite both of these factors being of significant interest in tumour development. However, inflammation was previously shown to increase *CYP1B1* expression in brain cells (Malaplate-Armand *et al*, 2003). In a recent study, IL6 was shown to induce *CYP1B1* but not *CYP1A1* gene expression in human hepatocellular carcinoma cell line HepG2 in an AhR-independent manner (Kurzawski *et al*, 2012). This is in line with our results, where IL6 induced CYP1B1 but not CYP1A1 in CRC cells and only CYP1B1 levels were significantly elevated in tumour tissue where IL6 expression was also increased. While Kurzawski *et al* (2012) did not establish a specific mechanism for the

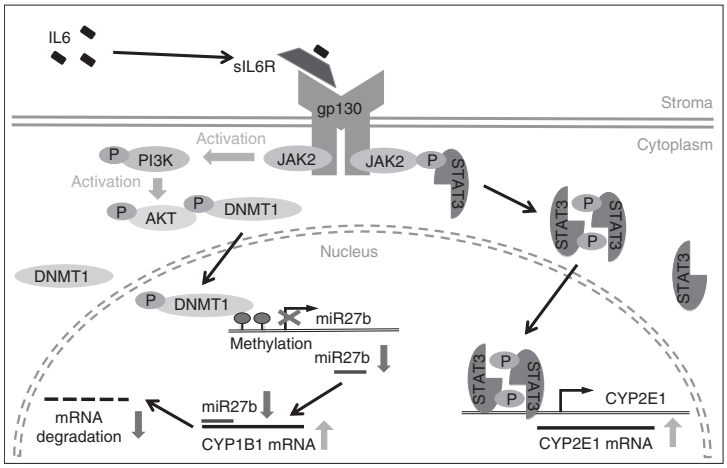

Figure 7. Potential mechanism of IL6-mediated induction of *CYP1B1* and *CYP2E1* mRNA expression in colorectal cancer cells. IL6 secreted by stromal cells binds to the soluble IL6 receptor (sIL6R) in the stroma, which interacts with transmembrane protein gp130 at the cellular surface resulting in activation of JAK/STAT3 and PI3K/AKT pathways. Activated STAT3 forms a homodimer, translocates to the nucleus and binds to the CYP2E1 promoter region, thus inducing its transcription. Activated AKT phosphorylates DNMT1 leading to its nuclear translocation. Once in the nucleus, DNMT1 induces DNA methylation at a CpG island located near miR27b, thus preventing its expression. Lower expression of miR27b results in less binding to the CYP1B1 mRNA leading to less degradation and increased CYP1B1 mRNA expression.

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induction of CYP1B1 by IL6, here we show that *CYP1B1* is post-transcriptionally regulated by IL6 through miR27b, an miRNA reported to directly target *CYP1B1* mRNA (Tsuchiya *et al*, 2006). Furthermore, our data suggest that DNA methylation has a role in IL6-mediated downregulation of miR27b. Yan *et al* (2011) reported a CpG island located near miR27b that is methylated in CRC cells and can regulate miR27b expression, thus supporting our findings. MiR27b has been shown to have various roles in the inflammatory process. In neuroblastoma cells, inhibition of miR27b activated NFκB signalling leading to increased expression of IL6 (Lee *et al*, 2011), whereas the opposite has been found in breast cancer cells (Jin *et al*, 2013). Despite these conflicting reports, miR27b and IL6 appear to be involved in the same pathways and this is the first account of IL6 directly regulating miR27b expression.

In summary, this study has shown that CYP450 enzymes expressed in colon tissue are significantly influenced by disease as evidenced by the increased expression of CYP2E1 and CYP1B1 in tumour tissue samples. However, it is important to note that a sample size of 40 patients was used in this study, and while our data are in agreement with previous reports of increased CYP1B1 expression, studies with larger patient numbers are needed to validate these findings, in particular for CYP2E1 expression. Furthermore, we noted the changes in CYP450 expression observed in malignant tissue did not correlate with sex or age. Interestingly, CYP450 expression was also not associated with tumour grade, suggesting that these changes occur at an early disease stage. We have previously shown these same colorectal tumours (from the same patient cohort) have increased levels of IL6 compared with adjacent normal tissues (Maihofner *et al*, 2003) and here we demonstrate for the first time that addition of IL6 can regulate *CYP1B1* and *CYP2E1* in colon tumour-derived cell lines. We describe a transcriptional mechanism for IL6-mediated *CYP2E1* induction *via* STAT3 (Figure 7). We also demonstrate an epigenetic mechanism by which IL6 represses miR27b expression involving DNA methylation resulting in *CYP1B1* upregulation (Figure 7). Taken together, our study provides further insight into the mechanisms by which IL6 promotes tumour development and progression. While altering CYP450 expression is only one of the mechanisms by which IL6 exerts its pro-tumourigenic effects, this pathway is of particular importance when it comes to designing therapies. New CRC treatment strategies could exploit this novel pathway potentially through the use of drugs that can only be activated by CYP2E1 or CYP1B1, and avoiding treatments that may be deactivated by these CYP450s. These findings also suggest that preventive dietary measures are of particular importance for patients with inflammatory bowel conditions to reduce their risk of CRC. By manipulating CYP450 enzymes, IL6 can induce phenotypic changes in colon tumour cells possibly rendering them drug resistant, or encouraging the *in situ* metabolism of carcinogens, thus resulting in DNA damage and potentially tumour promotion.

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Toxicology Research

PAPER

Interleukin-6 promotes dietary carcinogen-induced DNA damage in colorectal cancer cells

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Colorectal cancer (CRC) is the third most common cancer worldwide with 80% of cases being sporadic, arising following a series of environment-induced gene mutations. DNA damaging pro-carcinogens such as benzo[*a*]pyrene (BaP) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine (PhIP) contained in red or processed meats are a potential risk factor for disease. These dietary pro-carcinogens require metabolic activation to their genotoxic agents by cytochrome P450 (CYP) family 1 enzymes. We have previously demonstrated that the pro-inflammatory cytokine interleukin-6 (IL6) promotes *CYP1B1* expression in CRC cells grown as 2D monolayers and that these two proteins are overexpressed in malignant tissue resected from CRC patients, indicating that inflammation influences metabolic competency in CRC cells. To determine whether IL6 can influence BaP and PhIP activation, we investigated IL6 effect on BaP- and PhIP-induced DNA damage in CRC cell lines grown as 2D monolayers and as 3D spheroids using the *in vitro* micronucleus (MN) assay. We also investigated the involvement of p53 and CYPs in the observed effects. MN formation was increased dose-dependently following treatment with BaP and PhIP while pre-treatment with IL6 further enhanced DNA damage. We confirmed that IL6-mediated effects were not caused by p53 expression changes but rather by *CYP1B1* expression induction through miR27b down-regulation. Taken together, these data demonstrate that inflammatory cytokines can promote dietary pro-carcinogen activation and DNA damage in CRC cells.

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Introduction

As the colon is part of the digestive system, it is particularly susceptible to carcinogens present in the diet. Epidemiological studies have reported a significant correlation between high consumption of red and processed meats, known sources of dietary carcinogens, to increased incidence of CRC.[1]

Commonly occurring dietary carcinogens include benzo[*a*]-pyrene (BaP) and 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]-pyridine (PhIP).[2–4] BaP is a five-ring polycyclic aromatic hydrocarbon generated during the combustion of organic compounds and is commonly found in motor fumes, cigarette smoke and cooked meats.[5,6] It is thought to be involved in tobacco- and diet-associated cancers due to its mutagenic potential. PhIP is the most abundant heterocyclic amine found primarily in meats cooked at high temperatures, particularly fish, chicken and beef.[7] Studies have found correlations between meat consumption, PhIP intake and colon, breast and prostate cancers,[1,8–10] and PhIP-DNA adducts have been detected in these tissues,[11–13] supporting its role as a mutagen in these tissue types.

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Like many carcinogens, BaP and PhIP are activated into their genotoxic derivatives, 7,8-diol-9,10-epoxy BaP and *N*-hydroxy PhIP respectively, by CYP1 A1, A2 and B1 enzymes.[6,14,15] The activated molecules covalently bind DNA disrupting the double-helical structure resulting in DNA damage including double-strand breaks, deletions and points mutations.[6,16–19]

CYP1B1 and CYP1A1 are found extra-hepatically and are expressed in CRC tissue,[20–22] suggesting a potential for *in situ* metabolism and increased susceptibility to dietary carcinogen-induced mutations. However, mechanisms that regulate carcinogen activation in the colon are not yet well understood. Overexpression of pro-inflammatory cytokine interleukin-6 (IL6) occurs at the tumour site in CRC patients[23,24] and we recently determined that *CYP1B1* expression was epigenetically regulated by IL6 through miR27b in CRC cells grown as 2D monolayers.[20] Given our previous findings, IL6 may be involved in promoting activation of dietary pro-carcinogens in colonic cells.

In vitro techniques commonly use cells cultured as 2D monolayers. However, when cultured in this manner, cells are flattened onto the culture surface thus changing their structure, and parameters such as cell-to-cell interaction and tissue architecture are lost. *In vitro* 3D cell culture systems, in which cells are grown as spheroids, constitute a better model of

in vivo tissue without the use of animal models. Previous studies comparing 2D and 3D cell culture systems have shown 3D cell morphology and growth rate more accurately represent *in vivo* tumours.[25] Thus, 3D cell culture has become increasingly popular replacing standard cell culture techniques as a means of increasing *in vivo* relevance of *in vitro* experimental results.

In this study, we have adapted current *in vitro* techniques for use with 3D cultures and have compared results with that obtained with cells grown as 2D cultures. We have used this novel technique to investigate the mechanisms involved in diet-associated CRC by studying the effect of IL6 on BaP- and PhIP-induced DNA damage as well as underlying mechanisms for observed effects using *in vitro* models. Exploring these mechanisms is important to further understand the role of IL6 in diet-associated colorectal carcinogenesis and could potentially identify a novel regulator of dietary carcinogen activation.

Materials and methods

Cell culture

The human colorectal adenocarcinoma cell lines HCT116 and SW480 were obtained from ATCC (LGC Prochem, Middlesex, UK). HCT116 p53−/− cells were kindly provided by Professor Bert Vogelstein (John Hopkins University, Baltimore, MD, USA). Cells were routinely cultured in RPMI1640 medium (GIBCO, Life technologies, Paisley, UK) supplemented with 10% foetal bovine serum (FBS), 100 units per ml penicillin, 100 μg ml^{-1} streptomycin and 2 mM L-glutamine (GIBCO, Life technologies). All cells were incubated at 37 °C in a humidified incubator (5% CO_2). Cells between passages 3–7 were used for experiments.

For 3D cell culture, cells were seeded at a density of 5×10^5 cells per well in a 24-well Algimatrix system (Invitrogen, Life technologies) according to the manufacturer's protocol. Cells were monitored and culture medium was routinely changed. Spheroids cultured for 7–10 days were used in all experiments. To isolate spheroids from the matrix, matrix-dissolving buffer (Invitrogen, Life technologies) was used according to manufacturer's protocol.

Cell treatments

Prior to treatment, HCT116 and SW480 were maintained in culture medium supplemented with 5% dextran-coated charcoal-stripped FBS for at least 72 hours. Cells were seeded at a density of 1×10^5 cells per well of a 6-well plate (for 2D cell culture). HCT116 and SW480 do not express IL6 so human recombinant IL6 (HumanKine, Sigma-Aldrich, Dorset, UK) dissolved in PBS containing 0.1% human serum albumin (Sigma-Aldrich) was added to the cells for 24 and 48 hours at doses of 0–5000 pg ml^{-1} (chosen within the range secreted by stromal cells in the colon[26]).

For treatment with dietary carcinogens, cells were pre-treated with IL6 as described above, washed with PBS and treated with a dose-range of BaP (0–10 μM, Sigma-Aldrich) and PhIP (0–100 μM, Toronto Research Chemicals Inc., Toronto, Canada). Both chemicals were dissolved in DMSO and final vehicle control concentration of 0.2% was used.

Cytotoxicity and micronucleus assay

Micronucleus (MN) assay was performed according to OECD guidelines with modifications. Briefly, cells were seeded at a density of 5×10^4 cells per well of a 24-well plate for 2D cell culture. Spheroids grown for 10 days were used for 3D culture. Cells were treated with IL6, BaP or PhIP as detailed previously, washed with PBS and cultured for a further 72 hours prior to harvest. Etoposide (Sigma-Aldrich) was used as a positive control in all assays at a concentration of 125 nM. Cells were then harvested, resuspended in culture medium containing 2% pluronic (GIBCO, Life technologies) and cytotoxicity was determined by counting cells in a haemocytometer with Trypan-Blue exclusion (GIBCO, Life technologies). Cells were fixed with 100% methanol onto microscope slides at a density of 2×10^4 cells per slide and stained for 60 seconds with acridine orange (0.1 mg ml^{-1} dissolved in PBS, Sigma-Aldrich). Frequency of MN was scored blind in 1000 cells per sample and three biological replicates were performed per treatment.

RNA extraction

Total RNA was isolated from cells using TRIzol reagent (Invitrogen, Life technologies) according to the manufacturer's protocol. RNA extracts were quantified by UV spectroscopy (UV-VIS Nano-spectrophotometer, Implen, Essex, UK) with purity assessed from 260/280 nm and 260/230 nm ratios. Extracts were stored at −80 °C until used.

Reverse transcription and qPCR

Reverse transcription and qPCR were performed as previously described.[20] Briefly, for mRNA reverse transcription, Superscript II reverse transcription kit was used (Invitrogen, Life technologies) and miRNA reverse transcription kit was used for miRNA expression (Applied Biosystems, Life technologies). QPCR was performed using pre-designed expression assays (Taqman, Applied Biosystems, Life technologies) for *CYP1A1* (Hs01054797_g1), *CYP1B1* (Hs00164383_m1), *p53* (Hs01034249_m1), *GAPDH* (Hs99999905_m1), U6 (001973) and miR27b (000409). FAST PCR master mix was used according to the manufacturer's protocol (Taqman, Applied Biosystems, Life technologies).

Statistical analysis

Data were obtained from measurements made in at least three biological replicates and presented as a mean ± standard error (SEM). Significant differences ($p < 0.05$) were determined using Student's *t*-test, one-way analysis of variance (ANOVA) followed by a Dunnett post-test. Pearson's product-moment correlation coefficient test was used for correlation analysis (GraphPad Prism 5, GraphPad Software Inc., La Jolla, CA, USA).

Results

IL6 effect on chemical-induced DNA damage by food-derived pro-carcinogens BaP and PhIP

The *in vitro* MN assay is a commonly used toxicological test for detecting genotoxic potential of compounds due to its simplicity of scoring, accuracy and adaptability to different cell types. More importantly, studies in humans have shown strong associations between micronuclei frequency and cancer risk.[27] Formation of micronuclei, *i.e.* small membrane-bound DNA fragments in the cytoplasm, occurs during cell division when a whole chromosome or a chromosomal fragment is not incorporated into the nucleus of one of the daughter cells. Standard MN assays utilise immature blood cells, but non-standard versions of the assay have been developed using different cell types including epithelial cells. Here, we have adapted the assay for use with human colorectal adenocarcinoma epithelial cell lines grown as 2D and 3D cultures.

Cytotoxicity. Cytotoxicity was measured following IL6 and carcinogen treatment to ensure cell viability post-treatment. While some toxicity (around 30% drop in cell survival) was observed particularly with IL6 + BaP treatment in both cell lines, these differences were not statistically significant compared to respective controls (Fig. 1).

Genotoxicity. A dose-dependent increase in BaP- and PhIP-induced MN frequency was observed in HCT116 and SW480 cell lines cultured as 2D monolayers. Interestingly, pre-treatment with IL6 significantly enhanced the pro-carcinogen-induced DNA damage while treatment with IL6 on its own had no effect (Fig. 2). Etoposide, a topoisomerase II inhibitor and potent inducer of MN formation, was used as a positive control and does not require activation to induce DNA damage. Pre-treatment with IL6 did not enhance etoposide-induced DNA damage suggesting that IL6 may have an effect on the activation pathway of the pro-carcinogens rather than on MN formation itself.

3D cell culture. The assay was repeated using HCT116 cells grown as 3D spheroids. In general, results (Fig. 3) were in agreement with that found in 2D: no significant cytotoxicity was observed and pre-treatment with IL6 enhanced BaP and PhIP induced DNA damage. However, the increase in DNA damage observed with IL6 pre-treatment was not as pronounced in 3D culture compared to 2D: MN frequency was increased by 1.5 fold in 3D culture with IL6 pre-treatment

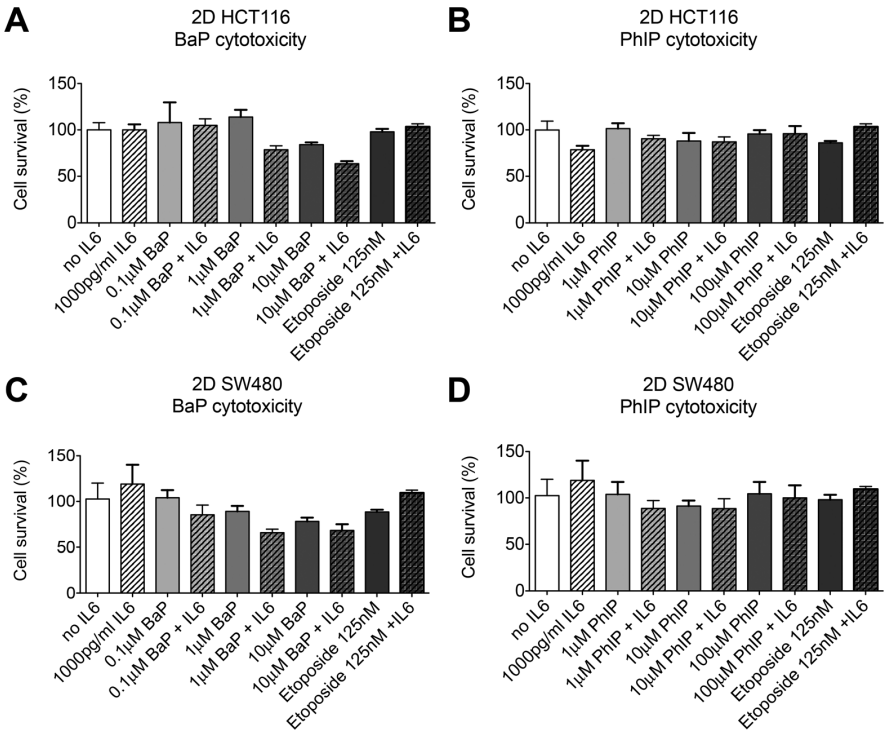

Fig. 1 Cytotoxicity of 2D HCT116 and SW480 induced by BaP and PhIP. HCT116 (A, B) and SW480 (C, D) cells grown as monolayers were pre-treated with IL6 for 48 hours and 24 hours respectively, followed by 24 hour treatment with BaP or PhIP. Cells were harvested 72 hours post-treatment. Cytotoxicity following treatment expressed as % of cell survival. Statistically significant differences were calculated using one-way ANOVA with a Dunnett post-test (GraphPad Prism 5). Error bars represent the SEM for independent cultures (n = 3).

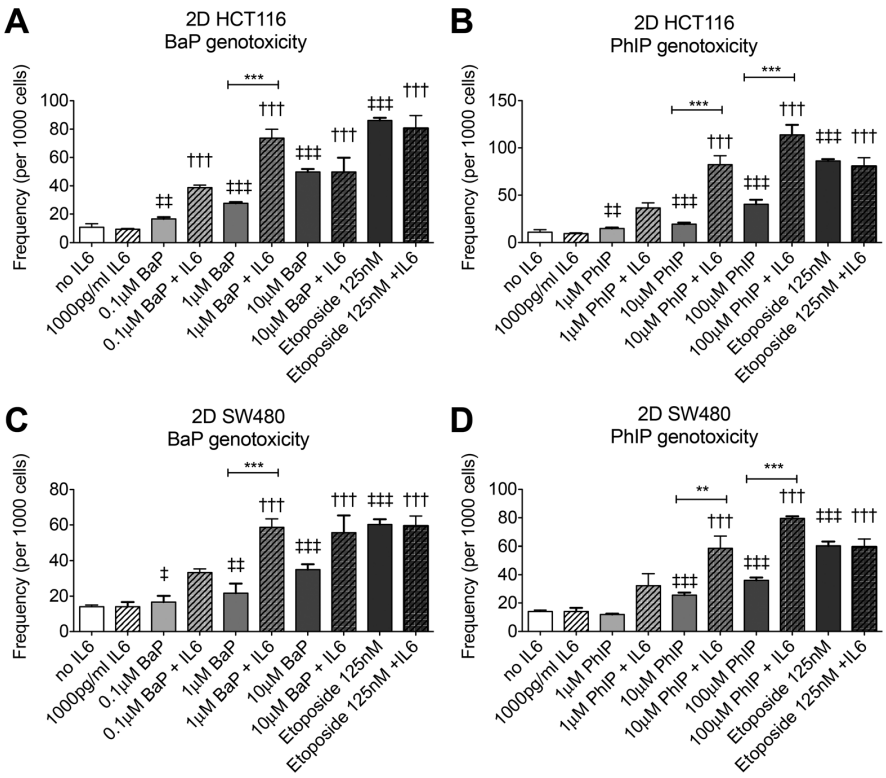

Fig. 2 DNA damage measured by MN frequency in 2D HCT116 and SW480 induced by BaP and PhIP. HCT116 (A, B) and SW480 (C, D) cells grown as 2D monolayers were pre-treated with IL6 for 24 hours followed by 24 hour treatment with BaP or PhIP. Cells were harvested 72 hours post-treatment. Etoposide was used as a positive control. Micronuclei (MN) frequency per 1000 cells was measured following treatment. Statistically significant differences are shown for comparisons between carcinogen treated *vs.* IL6 pre-treated samples (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$), IL6 alone *vs.* IL6 pre-treated and carcinogen treated (†††$p < 0.001$, ††$p < 0.01$, †$p < 0.05$) and vehicle *vs.* carcinogen treated (‡‡‡$p < 0.001$, ‡‡$p < 0.01$, ‡$p < 0.05$). Significance was calculated using one-way ANOVA with a Dunnett post-test (GraphPad Prism 5). Error bars represent the SEM for independent cultures ($n = 3$).

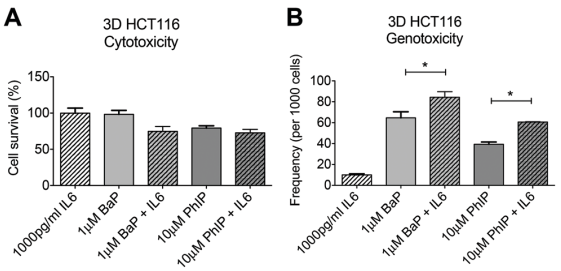

Fig. 3 Cytotoxicity and DNA damage in HCT116 grown as 3D spheroids. Cells grown as 3D spheroids on Algimatrix were pre-treated with IL6 for 24 hours followed by 24 hour treatment with BaP or PhIP. Cells were harvested 72 hours post-treatment. Etoposide was used as a positive control. (A) Cytotoxicity following treatment expressed as % of cell survival. (B) Micronuclei frequency per 1000 cells following treatment. Statistically significant differences are shown for comparisons between carcinogen treated *vs.* IL6 pre-treated samples (*$p < 0.05$). Significance was calculated using one-way ANOVA with a Dunnett post-test (GraphPad Prism 5). Error bars represent the SEM for independent cultures ($n = 3$).

while in 2D culture it was increased by 2–3 fold (Fig. 2). Taken together, these data demonstrate that presence of IL6 in colon epithelial cells along with food-derived pro-carcinogen can enhance induction of DNA damage. We therefore investigated the mechanism by which IL6 exerts this effect. IL6 is a pleiotropic cytokine with a multitude of functions; it has previously been shown to regulate *p53* gene expression[28] and *CYP1B1* expression,[20] thus we investigated whether these two pathways were responsible for IL6 mediated effects.

Mechanisms underlying IL6 effects

P53 involvement. The tumour suppressor protein p53 induces cell cycle arrest upon DNA damage recognition, activates DNA repair pathways or induces apoptosis in the compromised cell; p53 is thus crucial to maintain genomic stability. IL6 has been reported to induce downregulation of *p53* gene expression by promoting methylation of its promoter region.[28] Both of the cell lines used in this study have p53 activity. To test whether inactivation of p53 may be responsible

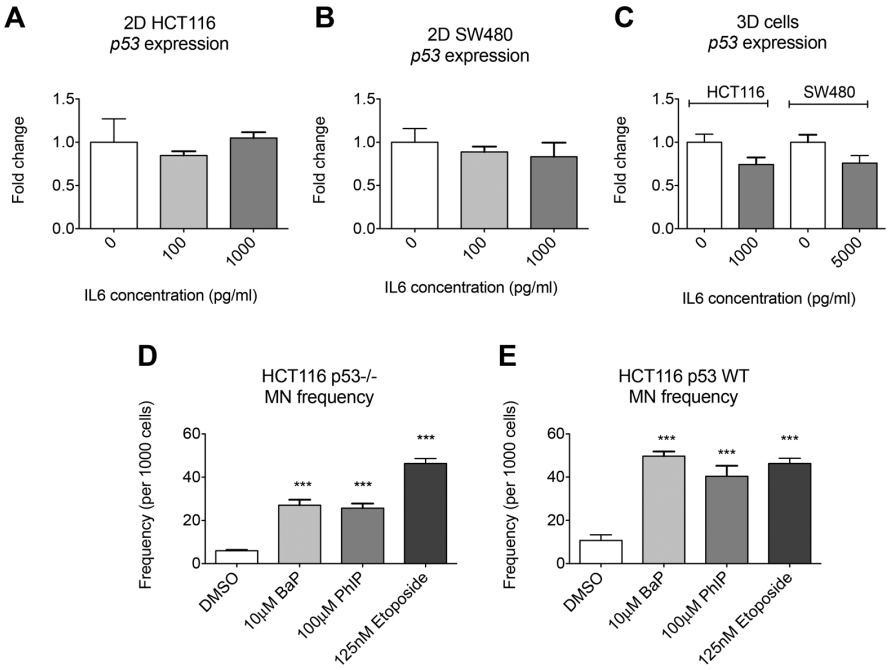

Fig. 4 Involvement of p53 in IL6-mediated induction of BaP- or PhIP-mediated DNA damage. HCT116 and SW480 cells grown as 2D monolayers (A, B) and 3D spheroids (C) were treated with IL6 and *p53* expression was measured by RT-qPCR. Data were normalised to expression of *GAPDH* and are show relative to control. (D–E) HCT116 p53−/− and HCT116 wild-type (WT) cells were grown as monolayers and treated for 24 hours with BaP or PhIP. Cells were taken 72 hours post-treatment and micronuclei frequency per 1000 cells were determined following treatment. Significance was calculated using one-way ANOVA with Dunnett post-test comparing treated group to vehicle control (GraphPad Prism 5, ***$p < 0.001$). Error bars represent the SEM for independent cultures ($n = 3$).

for the IL6 effect observed, we performed a MN assay on HCT116 p53−/− cells obtained from Professor Vogelstein's laboratory (John Hopkins University, Baltimore, MD, USA) and looked at the effect of IL6 treatment on *p53* gene expression by qPCR. No significant downregulation of *p53* gene expression was observed at the dose of IL6 used in this study (Fig. 4A–C). Previous studies used supraphysiological doses of IL6 (10 ng ml^{-1} (ref. 28)) and therefore, the dose used here (physiological levels) are likely to be too low to inhibit *p53* expression. In addition, MN frequency was not increased in the p53-null HCT116 cells when compared to wild-type HCT116, rather total number of MN was decreased (Fig. 4D and E). This demonstrates that the observed effect of IL6 on MN induction by BaP and PhIP is not caused by downregulation of *p53*, further suggesting that IL6 may affect the activation pathway of the pro-carcinogens *via* induction of *CYP1B1* expression as previously described.[20]

CYP450 involvement. Previous studies in this laboratory have demonstrated that IL6 can regulate *CYP1B1* expression *via* miR27b downregulation but not *CYP1A1* in CRC cells grown as 2D monolayers,[20] indicating that IL6 may be associated with a change in metabolic competency. In the current study, we examined the effect of IL6 treatment on *CYP450* expression in CRC cells grown as 3D spheroids to confirm our previous findings. As expected, treatment with IL6 did not alter *CYP1A1* expression (Fig. 5A). Upregulation of *CYP1B1* in both HCT116 and SW480 3D spheroids following IL6 treatment was observed along with downregulation of miR27b (Fig. 5B and C), thus confirming our previous observations in 2D culture.[20] Therefore IL6 regulates *CYP1B1* expression by downregulating miR27b.

Furthermore, we investigated the effect of 24–48 hour pre-treatment with IL6 followed by removal of the IL6 media then 24 hour BaP or PhIP treatment on *CYP1A1*, *CYP1B1* and miR27b expression. However, we found no significant changes with IL6 pre-treatment followed by BaP or PhIP compared to BaP or PhIP treatment alone (Fig. 6), likely due to the fact that IL6 is removed prior to BaP and PhIP treatment. Therefore, *CYP1B1* induction is not sustained following IL6 removal. These data suggest that the initial induction of *CYP1B1* expression (in the first 24 to 48 hours) by IL6 treatment is responsible for increased carcinogen activation and DNA damage.

BaP (but not PhIP) is a known inducer of the AhR pathway that controls *CYP1A1* and *CYP1B1* expression and here as expected, BaP significantly induced expression of both *CYP1B1* and *CYP1A1*. Furthermore, miR27b expression is not altered following BaP and PhIP treatment, indicating that unlike IL6,

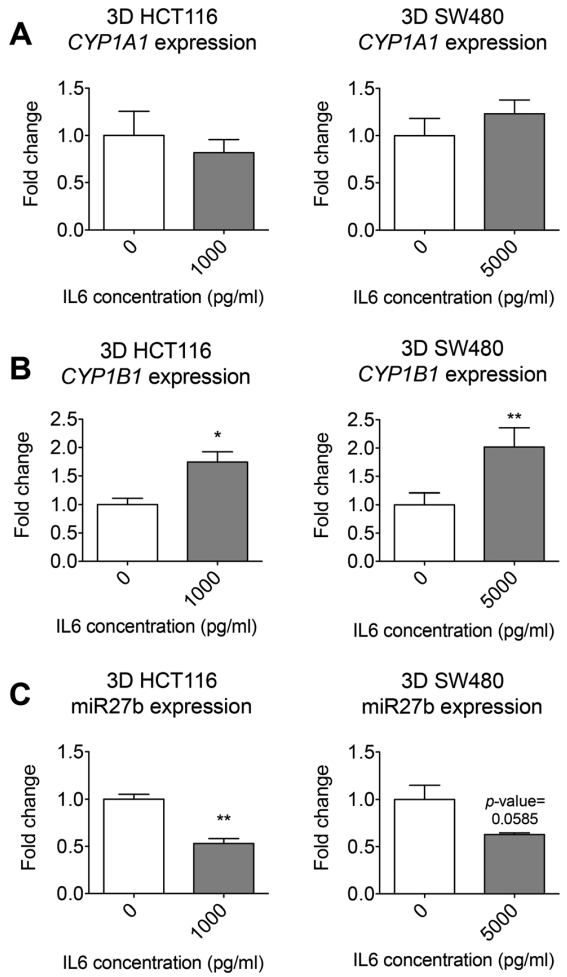

Fig. 5 IL6 effect on *CYP1A1*, *CYP1B1* and miR27b expression in 3D cultures. HCT116 and SW480 cells grown as 3D spheroids were treated with 0, 1000 or 5000 pg ml^{-1} IL6 for 24 hours. *CYP1A1* (A), *CYP1B1* (B) and *miR27b* (C) expression were measured by RT-qPCR. Data were normalised to expression of *GAPDH* for gene expression or U6 RNA for miRNA expression and are shown relative to control. Significance was assessed using Student's *t*-test comparing the treated group to vehicle control (GraphPad Prism 5, $^{**}p < 0.01$, $^{*}p < 0.05$). Error bars represent the SEM for independent cultures ($n = 3$).

BaP regulates *CYP1B1* expression *via* the AhR pathway and not miR27b.

Discussion

In the current study, IL6 was shown for the first time to promote DNA damage induced by BaP and PhIP, two carcinogens present in meats cooked at high temperatures. Umannová *et al.* previously reported that TNFα, another pro-inflammatory cytokine, increased BaP-induced genotoxic damage in alveolar epithelial type II cells,[29] suggesting that other inflammatory cytokines may have a similar effect, however specific mechanisms were not presented. TNFα is a known inducer of IL6 and therefore, IL6 may have been involved in the effect reported by Umammova *et al.*[29] Here, we suggest that IL6 promotes BaP- and PhIP-induced genotoxicity through induction of *CYP1B1* expression.

Previous studies on IL6 regulation of CYP450 enzymes present conflicting reports. Generally IL6 is thought to have an inhibitory effect in hepatic cells,[30–33] however a few studies in other cell types have shown increased expression of CYP450 in response to IL6,[20,34–36] which are in agreement with the data presented here in CRC cells grown as 3D spheroids. As previously shown in 2D cultures,[20] we found that IL6 downregulated miR27b expression resulting in increased *CYP1B1* expression in 3D cultured CRC cells. Furthermore, we also determine that IL6 mediated regulation of *CYP1B1* was not sustained following IL6 removal and determined that the higher levels of CYP1B1 in the cells caused by IL6 pre-treatment were likely to be responsible for the increased activation of BaP and PhIP. Moreover, we determined that BaP and PhIP do not regulate miR27b expression.

BaP and PhIP are also prevalent in other environmental factors such as pollution or smoking. In addition, CYP1B1 can also activate other environmental carcinogens,[37–41] and smokers have been shown to have higher plasma levels of IL6 compared to non-smokers.[42] Thus this novel IL6-mediated carcinogen activation pathway could also be important in other environment-associated cancers such as lung, and preventing IL6-mediated expression of CYP450 s could potentially be an effective disease prevention strategy. Regular intake of NSAIDs has been associated with lower cancer risk including CRC, breast and lung, however the specific underlying mechanisms remain unclear[43–45] and prevention of IL6-mediated induction of *CYP1B1* expression may be responsible, at least in part, for this effect.

CYP450 enzymes are also involved in metabolising a wide array of drugs, thus identifying mechanisms of their regulation in tumours could have significant implications in cancer therapies. High levels of IL6 at the tumour site has been associated with multiple drug resistance in a variety of cancer types.[46,47] IL6-mediated induction of local CYP450 expression could be involved in this effect as these enzymes could be inactivating chemotherapeutic drugs, thus administrating selected drugs that are not inactivated by these enzymes or combining drugs with an anti-IL6 adjuvant therapy could potentially attenuate drug resistance.

The IL6-mediated effects were observed in different *in vitro* culture systems (2D and 3D) as well as in two distinct CRC cell lines (HCT116 and SW480), thus providing further validation of the current findings. When comparing different cell culture methods, we observed that IL6-mediated effects on promoting BaP- and PhIP-induced DNA damage were not as pronounced in 3D cultures as compared to 2D cultures. Furthermore, for SW480 cells, higher doses of IL6 were required in 3D culture in order to reproduce responses observed in 2D culture. It has generally been shown that a decrease in drug sensitivity is

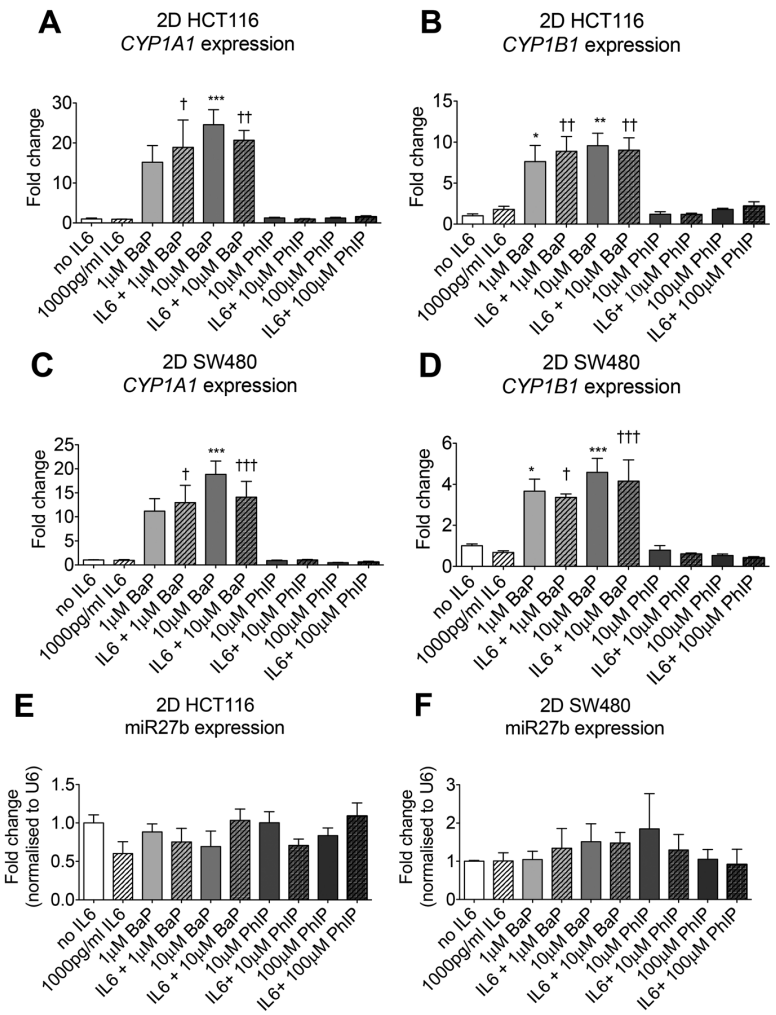

Fig. 6 *CYP1A1, CYP1B1* and miR27b expression following IL6 pre-treatment and BaP or PhIP treatment in 2D cultured HCT116 and SW480 cells. Cells grown as monolayers were pre-treated with 1000 pg ml^{-1} IL6 for 24 hours (SW480) or 48 hours (HCT116) followed by 24 hour treatment with BaP or PhIP. *CYP1A1* (A, C), *CYP1B1* (B, D) and *miR27b* (E, F) expression were measured by RT-qPCR. Data were normalised to expression of *GAPDH* for gene expression or U6 RNA for miRNA expression and are shown relative to control. Statistically significant differences were calculated using one-way ANOVA with a Dunnett post-test (GraphPad Prism 5) and are shown for comparisons between vehicle *vs.* carcinogen treated samples (***$p < 0.001$, **$p < 0.01$, *$p < 0.05$) and IL6 alone *vs.* IL6 pre-treated and carcinogen treated (†††$p < 0.001$, ††$p < 0.01$, †$p < 0.05$). Error bars represent the SEM for independent cultures ($n = 3$).

observed in cells cultured in 3D compared to 2D[48,49] likely due to the differences in level of exposure caused by the architecture of the spheroid, thus 3D culture better recapitulates *in vivo* responses.

Conclusion

In the current study, we investigated the effect of IL6 on DNA damage caused by dietary pro-carcinogens BaP and PhIP in CRC cells grown as 2D monolayers and 3D spheroids. Pre-treatment with IL6 enhanced BaP- and PhIP-induced DNA damage by promoting the activation of the carcinogens through *CYP1B1* expression induction. Increased levels of CYP1B1 in the cell along with presence of dietary carcinogens would lead to increased quantities of genotoxic metabolites, thus resulting in DNA damage. Discovery of this novel pathway provides further understanding of the mechanisms regulating dietary carcinogen activation in colonic cells and provides a mechanistic basis for the established chemopreventive activity of non-steroidal anti-inflammatory drugs in chemical-mediated colorectal carcinogenesis.[50]

Appendix D

Conflict of Interest

None declared.

Acknowledgements

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Appendix E: Conference Abstracts

1. Patel, S., Ellis, J., David, R. M. and Gooderham, N. J., 2014. Behavioural changes in human colorectal cancer cell lines grown as 3D spheroids. *Goodbye Flat Biology: 3D Models and the TME (EACR Conference Series)*, Berlin, Germany.

2. Patel, S., David, R. M. and Gooderham, N. J., 2014. IL6 can induce epigenetic change in colonic cells grown as 2D and 3D cultures resulting in increased activation of dietary carcinogens and DNA damage. *SOT annual meeting*, Phoenix, Arizona, USA.

3. Patel, S. and Gooderham, N. J., 2013. IL-6 can induce CYP1B1 expression through repression of miR27b resulting in enhanced dietary pro-carcinogen-induced DNA damage in colorectal cancer cells. *ICEM annual meeting*, Fos do Iguassu, Brasil.

4. Patel, S. and Gooderham, N. J., 2013. Interleukin-6 induces metastatic properties and cytochrome P450 expression in colorectal cancer cells. *BTS annual meeting*, Solihull, UK.

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